Remarks:

Claims:

By the present amendment, claims 61-67 have been amended to more particularly and distinctly define the invention. The number of total claims and of independent claims remains less than the amount for which fees were previously paid.

Claims 61 and 65 have been amended to comport with the restriction requirement of Paper No. 8.

Support for the amendments is either apparent, or is as described below. Support for "recombinant polypeptide" can be found, for example, at page 4, line 2; at page 8, line 27 through page 9, line 2; and at page 18, lines 19-21. No new matter is added.

Reconsideration of the rejections is respectfully requested.

Claim Rejection - 35 U.S.C. §102(b) - Bartos et al.

Claims 61-64 stand rejected under 35 U.S.C. §102(b) based on an assertion that the claims are anticipated by Bartos et al. (J. Infec. Dis., 158, 1988, pp 761-765). In particular the Examiner asserted:

> Bartos et al disclose an isolated polypeptide comprising member selected from outer membrane proteins i.e., OMP from whole cell lysate ATCC strain and other clinical isolates of M. catarrhalis (page 762, left and figure 1). Applicant's use of the open-ended term "comprising" and "fragments in the claims fails to exclude unrecited steps or ingredients and leaves the claims open for inclusion of unspecified ingredients, even in major amounts. Therefore, the claims read on the disclosed isolated polypeptide, OMP from M. catarrhalis. The isolated OMP from M. catarrhalis inherently comprise the amino acid sequence as set forth in the SEQ ID NO:2 or 4 and fragments of SEQ ID NO:2 or 4 See In re Horvitz, 168 F.2d 522, 78 U.S.P.Q. 79 (C.C.P.A. 1948) and Ex parte Davis et al., 80 U.S.P.Q. 448 (PTO d. App. 1948). In the absence of evidence to the contrary the disclosed prior art outer membrane protein read on the claimed isolated polypeptide. Since the Office does not have the facilities for examining and comparing applicants' claimed isolated polypeptide comprising SEQ ID NO:2 with the polypeptide of the prior art, the burden is on applicant to show a novel or unobvious difference between the claimed product and the product of the prior art. See In re Best, 562 F.2d 1252, 195 USPQ 430 (CCPA 1977) and In re Fitzgerald et al., 205 U.S.P.Q. 594.

Without conceding the correctness of the rejection, Applicant has amended the claims to more particularly and distinctly claim the subject matter of his invention. It is submitted that the amended claims recite an isolated, recombinant polypeptide. The claimed isolate is not disclosed or suggested by the OMP preparations described in Bartos et al. Reconsideration and withdrawal of the rejection is respectfully requested.

Claim Rejection - 35 U.S.C. §102(b) - Bartos et al.

Claims 61-64 and 66-68 stand rejected under 35 U.S.C. §102(b) based on an assertion that the claims are anticipated by Helminen et al. (J. Infec. Dis., 170, 1994, pp 867-872). In particular the Examiner asserted:

> Helmininen et al, 1994 disclose outer membrane proteins i.e., OMP's prepared from M.catarrhalis cells by EDTA buffer method. Monoclonal antibodies were produced by immunizing mice (page 868, left column under production of Mabs) with OMPs. Applicant's use of the open-ended term "comprising" in claim 61 fails to exclude unrecited steps or ingredients and leaves the claims open for inclusion of unspecified ingredients, even in major amounts. Therefore, the claims read on the disclosed isolated polypeptide, OMPs from M.catarrhalis. As OMPs comprise many proteins together it would read on fusion protein comprising said peptides and one other Moraxella antigen. Since monoclonal antibodies were raised against OMPS by immunizing the mice with OMPs in a buffer, the examiner considers the OMPs in a buffer as an immunogenic composition comprising said polypeptide in a pharmaceutically acceptable carrier. Therefore in the absence of evidence to the contrary the disclosed prior art OMPs read on the claimed invention. See In re Horvitz, 168 F.2d 522, 78 U.S.P.Q. 79 (C.C.P.A. 1948) and Ex parte Davis et al., 80 U.S.P.Q. 448 (PTO d. App. 1948). Since the Office does not have the facilities for examining and comparing applicants' claimed isolated polypeptide comprising SEQ ID NO:2 with the OMPs of the prior art, the burden is on applicant to show a novel or unobvious difference between the claimed product and the product of the prior art. See In re Best, 562 F.2d 1252, 195 USPQ 430 (CCPA 1977) and In re Fitzgerald et al., 205 U.S.P.Q. 594.

Without conceding the correctness of the rejection, Applicant has amended the claims to more particularly and distinctly claim the subject matter of his invention. It is submitted that the amended claims recite an isolated, recombinant polypeptide. The claimed isolate is not disclosed or suggested by the OMP preparations described in Helminen et al.

Moreover, Applicant notes that a later published paper, Aebi et al. (Infect. Immun. 65, 4367-4377) concurrently submitted as Exhibit A discloses the deduced amino acid sequences for the UspA1 and UspA2 genes. The two disclosed proteins are said to be reactive to the monoclonal antibody disclosed in Helminen et al. The proteins have accession numbers AAB96359 (UspA1) and AAB96391 (UspA2), and PubMed Sequence Viewer printouts are submitted as Exhibit B. Applicant submits that the disclosed sequences have no significant similarity to the claimed SEQ ID NO:2.

Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. §102(b) is respectfully requested.

Amendments to the Specification:

Entry of the amendments at page 4 (and the accompanying heading) and at page 67 into the specification is respectfully requested. The amendments to the specification are made to bring the specification in conformance with MPEP 608.01(a). Support for the amendments to page 4 can be found in the drawings as originally filed. No new matter is added.

Amendments to the Drawing Figures:

Replacement of the figures of record in the application with the concurrently filed replacement figures is respectfully requested. The figures have been amended to comply with 37 CFR 1.84. No new matter has been added.

Figures 1-8 have been amended to remove the title text, the description of which have been inserted in the Brief Description of the Drawings (see above). Figure 1 has been relabeled as Figures 1A-1L. Figure 2 has been relabeled as Figures 2A-2G. Figure 3 has been relabeled as Figure 3A-3C. Figure 7 has been relabled as Figures 7A-7B.

Information Disclosure Statement:

Applicant has concurrently filed an Information Disclosure Statement (IDS) listing the references cited in the International Search Report for PCT/EP99/03822 on a PTO-1449 form. It is noted that copies of the references have been received by the Office as indicated on the PTO Form entitled, "Notice of Acceptance of Application under 35 U.S.C. 371 and 37 CFR 1.494 or 1.495". It is respectfully requested that the listed references be included in the "References Cited" portion of any patent issuing from this application.

FEE DEFICIENCY

☒ If an extension of time is deemed required for consideration of this paper, please consider this paper to comprise a petition for such an extension of time; The Commissioner is hereby authorized to charge the fee for any such extension to Deposit Account No. 50-0258.

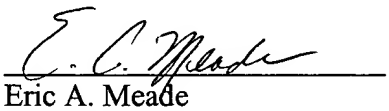
and/or

☒ If any additional fee is required for consideration of this paper, please charge Account No. 50-0258.

Closing Remarks

Applicant thanks the Examiner for the Office Action and believe this response to be a full and complete response to such Office Action. Accordingly, favorable reconsideration in view of this response and allowance of the pending claims are earnestly solicited.

Respectfully submitted,



Eric A. Meade
Registration No. 42,876
for
Allen Bloom
Registration No. 29,135
Attorney for Applicant

DECHERT LLP
A Pennsylvania Limited Liability Partnership
Princeton Pike Corporate Center
PO Box 5218
Princeton, New Jersey 08543-5218
Phone: (609) 620-3248
Fax: (609) 620-3259

INFECTION AND IMMUNITY, Nov. 1997, p. 4367–4377
0019-9567/97/$04.00+0

Vol. 65, No. 11

A Protective Epitope of *Moraxella catarrhalis* Is Encoded by Two Different Genes

CHRISTOPH AEBI,[1,2] ISOBEL MACIVER,[1] JO L. LATIMER,[1] LESLIE D. COPE,[1] MARLA K. STEVENS,[1] SHARON E. THOMAS,[1] GEORGE H. McCRACKEN, JR.,[2] AND ERIC J. HANSEN[1]*

Departments of Microbiology[1] and Pediatrics,[2] University of Texas Southwestern Medical Center, Dallas, Texas 75235-9048

Received 11 March 1997/Returned for modification 15 May 1997/Accepted 14 July 1997

The high-molecular-weight UspA protein of *Moraxella catarrhalis* has been described as being both present on the surface of all *M. catarrhalis* disease isolates examined to date and a target for a monoclonal antibody (MAb 17C7) which enhanced pulmonary clearance of this organism in a mouse model system (M. E. Helminen et al., J. Infect. Dis. 170:867–872, 1994). A recombinant bacteriophage that formed plaques which bound MAb 17C7 was shown to contain a *M. catarrhalis* gene, designated *uspA1*, that encoded a protein with a calculated molecular weight of 88,271. Characterization of an isogenic *uspA1* mutant revealed that elimination of expression of UspA1 did not eliminate the reactivity of *M. catarrhalis* with MAb 17C7. In addition, N-terminal amino acid analysis of internal peptides derived from native UspA protein and Southern blot analysis of *M. catarrhalis* chromosomal DNA suggested the existence of a second UspA-like protein. A combination of epitope mapping and ligation-based PCR methods identified a second *M. catarrhalis* gene, designated *uspA2*, which also encoded the MAb 17C7-reactive epitope. The UspA2 protein had a calculated molecular weight of 62,483. Both the isogenic *uspA1* mutant and an isogenic *uspA2* mutant possessed the ability to express a very-high-molecular-weight antigen that bound MAb 17C7. Southern blot analysis indicated that disease isolates of *M. catarrhalis* likely possess both *uspA1* and *uspA2* genes. Both UspA1 and UspA2 most closely resembled adhesins produced by other bacterial pathogens.

The three most important causes of acute otitis media are *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae*, and *Moraxella catarrhalis*. *S. pneumoniae*, the most prevalent cause of middle ear disease, is responsible for at least 30% of these infections (15), whereas *M. catarrhalis* and nontypeable *H. influenzae* each account for approximately 15 to 26% of middle ear infections (5, 6, 36, 51, 57, 67). While the pathogenic potential of nontypeable *H. influenzae* strains has been recognized for some time (45), only recently has *M. catarrhalis* emerged as a significant cause of respiratory tract disease (6). Formerly named both *Branhamella catarrhalis* and *Neisseria catarrhalis*, this organism was long considered a nonpathogenic, commensal inhabitant of the upper respiratory tract (6). It is now accepted that *M. catarrhalis* is an important cause of otitis media in children. In fact, in a recent report, *M. catarrhalis* DNA could be detected by PCR in middle ear effusions from 46% of patients with chronic otitis media with effusion (51). *M. catarrhalis* also causes lower respiratory tract disease, including acute bronchitis and exacerbation of chronic bronchitis in adults, especially those with compromised respiratory function (23, 44, 49, 68).

The recent recognition of *M. catarrhalis* as an important pathogen in both the upper and lower respiratory tracts has resulted in increased interest in both its interactions with the human host (11–14, 17–19, 29, 35) and its antigenic composition. Outer membrane proteins constitute major antigenic determinants of this unencapsulated organism (3), and different strains share remarkably similar outer membrane protein profiles (3, 46). At least three different surface-exposed outer membrane antigens have been shown to be well conserved among *M. catarrhalis* strains; these include the 81-kDa CopB outer membrane protein (26), the heat-modifiable CD outer membrane protein (30, 47), and the very-high-molecular-weight UspA protein (27). Of these three antigens, both the CopB and UspA proteins have been shown to bind antibodies which exert biological activity (i.e., protection) against *M. catarrhalis* in an animal model (27, 47).

Previous studies revealed that at least one epitope of the UspA protein is exposed on the surface of all disease isolates of *M. catarrhalis* tested to date; this epitope is defined by its reactivity with the protective monoclonal antibody (MAb) 17C7 (27). The UspA protein of *M. catarrhalis* 035E migrates with an apparent molecular weight of at least 250,000, and the UspA proteins of other *M. catarrhalis* strains appear to be even larger (27, 34). In the present study, a *M. catarrhalis* strain 035E gene encoding a MAb 17C7-reactive protein was shown to encode an 88-kDa protein, designated UspA1, that contained a number of amino acid repeat motifs. Mutant analysis revealed that inactivation of the expression of the *uspA1* gene did not eliminate the reactivity of *M. catarrhalis* 035E with MAb 17C7. A combination of epitope mapping and PCR technology was used to identify a second *M. catarrhalis* gene encoding a 62-kDa protein, designated UspA2, which also contained the MAb 17C7-reactive epitope.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *M. catarrhalis* 035E, TTA24, and P44 have been described previously (26, 27, 65). *M. catarrhalis* TTA1 and TTA37 were obtained from transtracheal aspirates and provided by Steven Berk, East Tennessee State University, Johnson City. *M. catarrhalis* 25240 was obtained from the American Type Culture Collection, Rockville, Md. *M. catarrhalis* strains were routinely cultured at 37°C in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) or on BHI agar plates in an atmosphere of 95% air–5% CO_2. When appropriate, kanamycin was added to the BHI medium to a final concentration of 20 μg/ml. *Escherichia coli* DH5α, LE392, and XL1-Blue MRF′ (Stratagene, La Jolla, Calif.) were grown on Luria-Bertani medium (41) at

* Corresponding author. Mailing address: Department of Microbiology, Hamon Building, NA6.200, University of Texas Southwestern Medical Center, 6000 Harry Hines Blvd., Dallas, TX 75235-9048. Phone: (214) 648-5974. Fax: (214) 648-5905. E-mail: hansen01@utsw.swmed.edu.

TABLE 1. Bacteriophages and plasmids used in this study

Bacteriophage or plasmid	Description	Source
Bacteriophages		
LambdaGEM-11	Cloning vector	Promega Corp.
MEH200	LambdaGEM-11 containing an 11-kb insert of *M. catarrhalis* 035E DNA encoding the UspA1 protein	27
ZAP Express	Cloning vector	Stratagene
USP100	ZAP Express with a 2.7-kb fragment of DNA (containing *uspA1*) amplified from the chromosome of *M. catarrhalis* 035E	This study
USP200	ZAP Express with a 2.1-kb fragment of DNA (containing *uspA2*) amplified from the chromosome of *M. catarrhalis* 035E	This study
Plasmids		
pBS	Cloning vector, Amp^r	Stratagene
pJL501.6	pBS containing the 1.6-kb *BglII-EcoRI* fragment from MEH200	This study
pJL500.5	pBS containing the 600-bp *BglII* fragment from MEH200	This study
pUSPA1	pBS containing the partial *uspA1* ORF (bp 339 to 2984)	This study
pUSPA1KAN	pUSPA1 with a *kan* cartridge inserted into the partial *uspA1* ORF	This study
pGEX-4T-2	GST fusion protein vector	Pharmacia
pMF-3	pGEX-4T-2 containing bp 1185 to 1742 from *uspA1*	This study
pMF-4	pGEX-4T-2 containing bp 1638 to 2303 from *uspA1*	This study
pMF-4-1	pGEX-4T-2 containing bp 1722 to 1954 from *uspA1*	This study
pMF-4-2	pGEX-4T-2 containing bp 1934 to 2303 from *uspA1*	This study
pUSPA2	pBS containing the partial *uspA2* ORF (bp 649 to 2596)	This study
pUSPA2KAN	pUSPA2 with a *kan* cartridge inserted into the partial *uspA2* ORF	This study

37°C, supplemented with maltose (0.2%, wt/vol), 10 mM $MgSO_4$, and antimicrobial agents as necessary.

MAbs and immunological methods. MAb 17C7 is a murine immunoglobulin G (IgG) antibody reactive with the UspA protein of all *M. catarrhalis* disease isolates tested to date (27). This MAb was used in the form of hybridoma culture supernatant fluid in all experiments. The colony blot-radioimmunoassay has been described before (22).

Cloning vectors. Plasmid and bacteriophage cloning vectors utilized in this study and the recombinant derivatives of these vectors are listed in Table 1. MEH200, the original recombinant bacteriophage clone that produced plaques reactive with the UspA-specific MAb 17C7, has been described previously (27).

Genetic techniques. Standard recombinant DNA techniques, including plasmid isolation, restriction enzyme digestions, DNA modifications, ligation reactions, and transformation of *E. coli*, were performed as described previously (41, 53). The use of electroporation to construct isogenic mutants of *M. catarrhalis* has been described (28); the 1.2-kb *kan* cartridge used for these experiments was excised from pUC4K (Pharmacia-LKB Biotechnology, Piscataway, N.J.) by digestion with *Bam*HI.

Southern blot analysis. Southern blot analysis of chromosomal DNA fragments derived from *M. catarrhalis* strains was performed as described before (26). Oligonucleotide probes were labeled with a 3'-end labeling fluorescein kit (Dupont NEN, Wilmington, Dela.). Double-stranded DNA probes were labeled with ^{32}P with a random-primed DNA labeling kit (Boehringer-Mannheim, Indianapolis, Ind.).

PCR. The PCR was performed with the GeneAmp kit (Perkin-Elmer, Branchburg, N.J.). All reactions were carried out as described in the manufacturer's instructions. To amplify products from total genomic DNA, 1 μg of *M. catarrhalis* chromosomal DNA and 100 ng of each primer were used in each 100-μl reaction mixture. Ligation-based PCR experiments (i.e., vector-anchored PCR) (20, 37) were performed essentially as described before (33), except as noted. Oligonucleotide primers used in this study were designated P1 to P18 in Fig. 2 and 6.

Nucleotide sequence analysis. Nucleotide sequence analysis of DNA fragments in recombinant plasmids or derived from PCR was performed with an Applied Biosystems (Foster City, Calif.) model 373A automated DNA sequencer. Nucleotide sequence analysis of a *M. catarrhalis* DNA insert in a bacteriophage was facilitated by use of a presequencing kit for linear double-stranded DNA (United States Biochemicals, Cleveland, Ohio); this DNA was analyzed by double-stranded sequencing methods (70). Nucleotide sequence information was analyzed with the Intelligenetics suite package and programs from the University of Wisconsin Genetics Computer Group sequence analysis package (version 8.1) (10). Analysis of protein hydrophilicity by the method of Kyte and Doolittle (38) and analysis of repeated amino acid sequences within proteins were performed with the MacVector 6.0 software protein matrix analysis package (Oxford Molecular Ltd., Campbell, Calif.). The GAP alignment algorithm contained in the University of Wisconsin software package was used for direct comparison of the amino acid sequences of entire proteins. The ClustalW program for pairwise alignment with the Blosum 30 scoring matrix, as contained in MacVector 6.0, was utilized to determine similarity between peptides.

Identification of recombinant bacteriophage. Lysates were generated from *Escherichia coli* cells infected with recombinant bacteriophage by use of the plate lysis method as described previously (27). MAb-based screening of plaques formed by recombinant ZAP Express bacteriophage on *E. coli* XL1-Blue MRF' cells was performed as described in the manufacturer's instructions. Briefly, nitrocellulose filters soaked in 10 mM IPTG (isopropyl-β-D-thiogalactopyranoside) were applied to the surface of agar plates 5 h after bacteriophage infection of the bacterial lawn. After overnight incubation at 37°C, the nitrocellulose pads were removed, washed with phosphate-buffered saline (PBS) containing 0.5% (vol/vol) Nonidet P-40 and 5% (wt/vol) skim milk (PBS-N), and incubated with hybridoma culture supernatant containing MAb 17C7 for 4 h at room temperature. After four washes with PBS-N, PBS-N containing ^{125}I-labeled goat anti-mouse IgG was applied to each pad. After overnight incubation at 4°C, the pads were washed four times with PBS-N, blotted dry, and exposed to film.

Characterization of *M. catarrhalis* protein antigens. Outer membrane vesicles were extracted from BHI broth-grown *M. catarrhalis* cells by the EDTA-buffer method (48). Proteins present in these vesicles were solubilized by heating at 100°C for 5 min in digestion buffer (21) and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 7.5% (wt/vol) polyacrylamide separating gels. These SDS-PAGE-resolved proteins were transferred electrophoretically to nitrocellulose, and Western blot (immunoblot) analysis was performed as described previously with MAb 17C7 as the primary antibody (32). Radioiodinated goat anti-mouse IgG was used as the secondary antibody.

N-terminal amino acid sequence analysis. Proteins present in outer membrane vesicles or cell envelopes (24) prepared from *M. catarrhalis* 035E were resolved by SDS-PAGE, and the UspA protein band was excised. This protein was electroeluted and then again subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane by the method of Matsudaira (42). The protein was then digested with trypsin, endoproteinase Lys-C (Promega), or cyanogen bromide. The resultant peptides were resolved by high-performance liquid chromatography and subjected to N-terminal amino acid sequence analysis as described before (25).

Construction and analysis of fusion proteins. A glutathione *S*-transferase (GST) fusion protein system was used for localization of the epitope in UspA1 that bound MAb 17C7. Pairs of oligonucleotide primers were designed to amplify 400- to 600-bp fragments spanning the *uspA1* gene from *M. catarrhalis* 035E. Each of these primers had either a *Bam*HI site or a *Xho*I site at the 5' end, thereby allowing directional in-frame cloning of the amplified product into the *Bam*HI- and *Xho*I-digested pGEX-4T-2 vector (Pharmacia). Each of the resultant plasmid constructs was confirmed by nucleotide sequence analysis. Whole-cell lysates prepared from each recombinant *E. coli* strain were probed in Western blot analysis independently with MAb 17C7 and with a polyclonal mouse antiserum specific for GST (to verify expression of the fusion protein). Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin was used as the secondary antibody.

Nucleotide sequence accession number. The complete nucleotide sequences of the *uspA1* and *uspA2* genes from *M. catarrhalis* 035E have been deposited in GenBank under accession numbers U57551 and U86135, respectively.

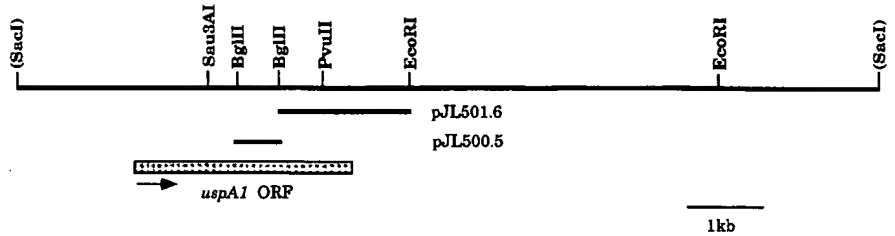


FIG. 1. Partial restriction enzyme map of the *M. catarrhalis* DNA insert in MEH200 and its derivatives. Relevant restriction sites are indicated; the *Sau*3AI site is not the only *Sau*3AI site in the insert. The 1.6-kb internal *Bgl*II-*Eco*RI fragment and the 600-bp internal *Bgl*II fragment were subcloned into pBS, creating the plasmids pJL501.6 and pJL500.5, respectively. The inserts in these plasmids are indicated by black bars. The shaded box indicates the position of the 2.5-kb *uspA1* ORF; the arrow beneath the left corner of this ORF indicates the direction of transcription. The use of parentheses around the *Sac*I sites indicates that these sites are in the vector DNA.

RESULTS

Subcloning and analysis of the *uspA1* gene. An 11-kb fragment of chromosomal DNA from *M. catarrhalis* 035E encoding a MAb 17C7-reactive protein was originally obtained from a bacteriophage-based genomic library (27). For the purpose of clarity, the *M. catarrhalis* gene encoding this MAb 17C7-reactive protein has now been designated *uspA1*. Efforts to subclone the entire 11-kb *M. catarrhalis* DNA insert from the recombinant bacteriophage MEH200 (Fig. 1) into a plasmid vector were unsuccessful, even when very-low-copy-number vectors (e.g., pLG338 [62]) were utilized for this purpose. When attempts were made to introduce various restriction fragments from this 11-kb insert into plasmid vectors, four fragments comprising 8.2 kb of contiguous DNA were subcloned successfully. These included the 600-bp *Bgl*II fragment, the 1.6-kb *Bgl*II-*Eco*RI fragment, the 4-kb *Eco*RI fragment, and the 2-kb *Eco*RI-*Sac*I fragment (Fig. 1). However, none of these recombinant clones expressed the MAb 17C7-reactive antigen.

Preliminary nucleotide sequence analysis of the 4-kb *Eco*RI and the 2-kb *Eco*RI-*Sac*I fragments revealed that these represented chimeras containing both *M. catarrhalis* DNA and *E. coli* DNA; no further analyses of these fragments were performed. In contrast, nucleotide sequence analysis of the 600-bp *Bgl*II fragment in the recombinant plasmid pJL500.5 and 1.6-kb *Bgl*II-*Eco*RI fragment in the recombinant plasmid pJL501.6 (Fig. 1) revealed the presence of a partial open reading frame (ORF) that encoded an incomplete 54-kDa polypeptide. The beginning of this partial ORF was inferred to be localized within the 2.8-kb *Sac*II-*Bgl*II fragment (Fig. 1).

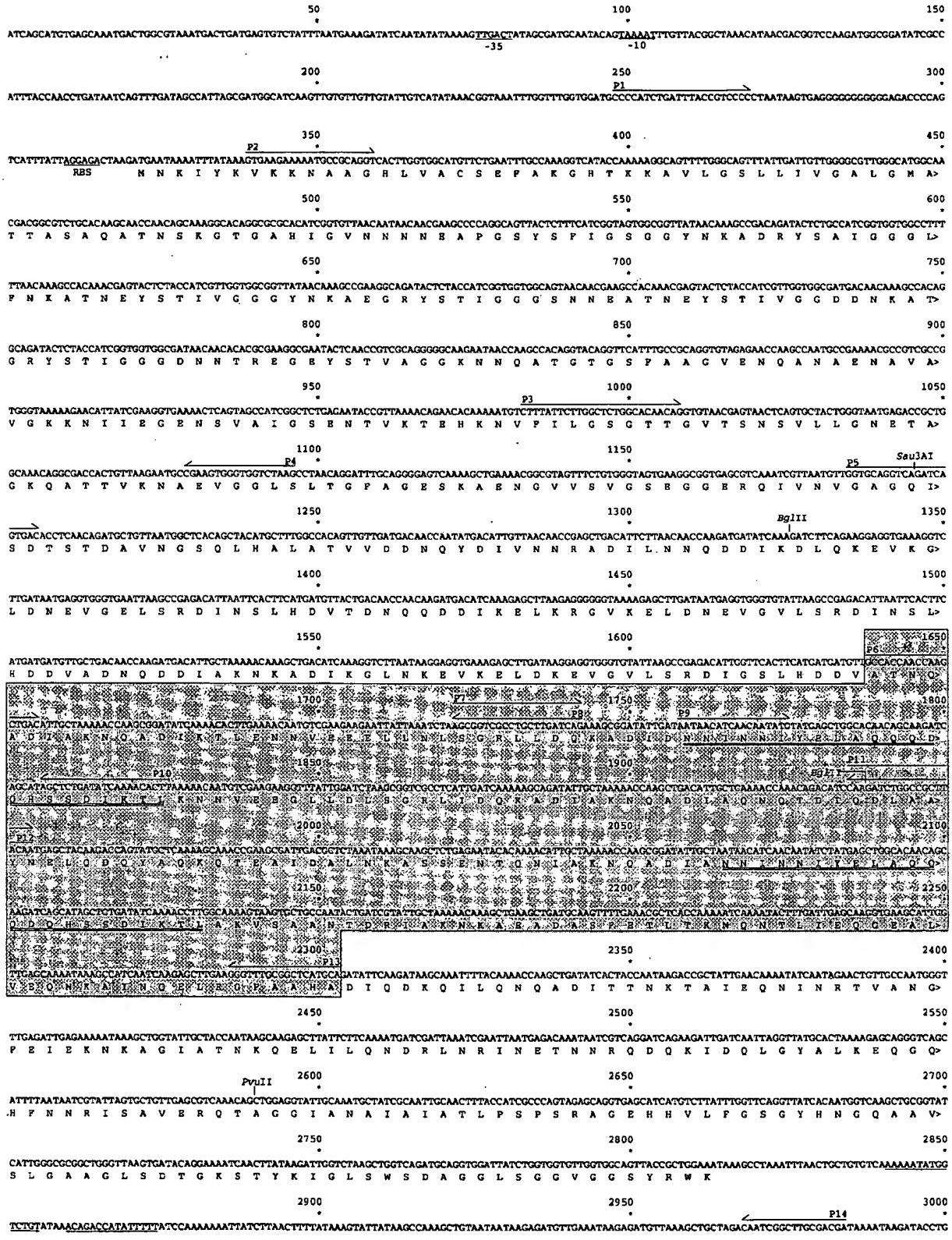
All attempts to subclone the 2.8-kb *Sac*I-*Bgl*II fragment were unsuccessful. However, double-stranded sequencing of the bacteriophage DNA, although very inefficient, yielded approximately 500 nucleotides of additional sequence 5′ from the first *Bgl*II site in MEH200; this new sequence included a *Sau*3AI site (Fig. 1). A 26-nucleotide probe specific for a region 5′ from this *Sau*3AI site in the MEH200 insert (see P3 in Fig. 2) was shown to hybridize to a 1.2-kb *Sau*3AI fragment from *M. catarrhalis* 035E by Southern blot analysis (data not shown). This allowed us to infer that this 1.2-kb *Sau*3AI fragment contained the 5′ end of the putative *uspA1* ORF.

Use of ligation-based PCR for nucleotide sequence analysis. *Sau*3AI-digested chromosomal DNA fragments from this strain were ligated into the *Bam*HI site in pBluescript II SK+ (pBS), and the ligation reaction mixture was precipitated, dried, and resuspended in 50 μl of sterile distilled water. This material was subjected to PCR amplification with an oligonucleotide primer specific for a region immediately 5′ from the relevant *Sau*3AI site in MEH200 (see P4 in Fig. 2) and a primer specific for the pBS T7 promoter. After PCR amplification, the entire reaction mixture was subjected to agarose gel electrophoresis. A 1.2-kb band (corresponding to the predicted size of the desired *Sau*3AI fragment) was obtained and used as the template in a second round of PCR amplification with the same primers.

Nucleotide sequence analysis of this final PCR product revealed that it contained the likely translational start site, together with a putative promoter region, to complete the partial ORF encoded by the inserts in the recombinant plasmids pJL501.6 and pJL500.5 (Fig. 1). The position of this ORF in the original *M. catarrhalis* DNA insert in MEH200 is shown in Fig. 1. Primers P1 and P14 (see Fig. 2) were used to amplify a 2.7-kb fragment containing the entire *uspA1* ORF directly from the chromosome of *M. catarrhalis* 035E, and both strands of this 2.7-kb PCR product were sequenced in their entirety to confirm the nucleotide sequence information derived from the ligation-based PCR product.

Features of the *uspA1* gene and its encoded protein product. The nucleotide sequence of the *M. catarrhalis* 035E *uspA1* gene and the deduced amino acid sequence of the UspA1 protein are shown in Fig. 2. The ORF, containing 2,496 nucleotides, encoded a protein product of 831 amino acids, with a calculated molecular weight of 88,271. The likely translational start site was located at nucleotide 321 (Fig. 2); this ATG start codon is located 7 nucleotides downstream from a sequence (5′-AGGA-3′) with homology to ribosome binding sites (56). Putative −10 and −35 consensus sequences were also identified upstream from the putative start codon (Fig. 2). A possible stem-loop terminator sequence was located between nucleotides 2841 and 2874. There were no ORFs located within 300 bp 5′ from the start of the *uspA1* gene. On the opposite DNA strand, approximately 200 bp from the 3′ end of the *uspA1* gene, there was an ORF encoding a predicted product similar to the *E. coli* P14 protein (52). The predicted protein product of the *uspA1* ORF was fairly hydrophilic and was distinguished by its high content of a number of different amino acid repeat motifs containing at least three predicted leucine zippers (data not shown). The significance of these motifs remains to be determined.

Similarity of UspA1 to other proteins. When the nucleotide sequence of *uspA1* was analyzed through the National Center for Biotechnology Information by use of the BLAST network service to search GenBank (2, 16), the *hsf* gene product of *H. influenzae* type b (61) was found to be the prokaryotic protein most similar to this *M. catarrhalis* antigen. This *H. influenzae* protein forms short, thin fibrils on the surface of *H. influenzae* type b that promote attachment to human epithelial cells (60). Other proteins retrieved from database searches as having


-35
-10
P1
P2
RBS
P3
P4
P5
Sau3AI
BglII
P6
P7
P8
P9
P10
P11
P12
P13
PvuII
P14

GAATGGACA

FIG. 2. Nucleotide sequence of the *uspA1* gene from *M. catarrhalis* 035E together with the deduced amino acid sequence. Putative −35 and −10 regions are indicated; a possible ribosome binding site (RBS) is also indicated. An inverted repeat located 3′ from the ORF is indicated by opposing arrows at nucleotides 2841 to 2874. Oligonucleotide primers (P1 to P14) used for PCR amplification are indicated by arrows placed above the relevant sequences. The shaded box contains the nucleotide sequence encoding the 222 amino acids present in the MF-4 fusion protein. The 23-residue peptides common to both the MF-4-1 and MF-4-2 fusion proteins are double underlined in this shaded box. Relevant restriction sites are indicated.

some similarity with UspA1 included myosin heavy chains from a number of species.

N-terminal amino acid sequence analysis of internal UspA peptides. To confirm the deduced amino acid sequence of the UspA1 protein, we performed N-terminal amino acid sequence analysis on peptide fragments from native UspA protein. The very-high-molecular-weight UspA protein (i.e., apparent molecular weight of greater than 250,000 in SDS-PAGE [27]) from *M. catarrhalis* 035E was resolved by SDS-PAGE, electroeluted, and digested with various proteinases or with cyanogen bromide. When the resultant peptides were subjected to N-terminal amino acid sequence analysis, several of the peptides exactly or closely matched peptides located near the center of the deduced amino acid sequence of the UspA1 protein (Table 2). However, four additional peptides with sequences that had only weak similarity to or were not present in the deduced amino acid sequence were found (Table 2). The degree of identity between the sequences of these four peptides and the deduced amino acid sequence of the UspA1 protein ranged from 26 to 33% (ClustalW score range, 35 to 43). These findings first raised the possibility that there might be a second protein, similar to UspA1, present in the electroeluted UspA protein band.

Southern blot analysis with a *uspA1* gene probe. To obtain preliminary genetic evidence for the possible existence of a second gene encoding a UspA1-like protein, we used a DNA fragment from the *uspA1* gene to probe chromosomal DNA from several *M. catarrhalis* strains. A 600-bp *Bgl*II-*Pvu*II fragment from pJL501.6, containing the 3′ end of the *uspA1* gene from strain 035E (Fig. 1), was used to probe a *Pvu*II digest of chromosomal DNA from strain 035E and five additional strains of *M. catarrhalis*. Interestingly, each strain yielded two *Pvu*II fragments that hybridized with this probe (Fig. 3, lanes A to F). This finding reinforced the possibility that there was another *uspA1*-like gene in the *M. catarrhalis* chromosome.

Construction and characterization of an isogenic *uspA1* mutant. Mutant analysis was utilized to determine conclusively whether there were two similar UspA proteins expressed by *M. catarrhalis*. Oligonucleotide primers (P2 and P14 [Fig. 2], each with a *Bam*HI site at the 5′ end) were used to amplify a 2.6-kb product containing most of the *uspA1* ORF except the first 18 nucleotides; this PCR product was cloned into the *Bam*HI site of pBS, yielding the recombinant plasmid pUSPA1 (Table 1). A 0.6-kb *Bgl*II fragment from the middle of this cloned fragment was excised and replaced by a *Bam*HI-ended *kan* cartridge. This new plasmid, designated pUSPA1KAN, was linearized by digestion with *Eco*RI and used to electroporate the wild-type *M. catarrhalis* strain 035E as described before (28). Approximately 5,000 kanamycin-resistant transformants were obtained; several picked at random were found to be still reactive with MAb 17C7 in the colony blot-radioimmunoassay. One of these kanamycin-resistant strains, designated *M. catarrhalis* 035E.1, was randomly chosen for further testing.

Southern blot analysis confirmed that 035E.1 was an isogenic *uspA1* mutant. When chromosomal DNA from both the wild-type parent strain and strain 035E.1 was digested with *Pvu*II and probed in Southern blot analysis with the 600-bp *Bgl*II-*Pvu*II fragment from pJL501.6, the wild-type strain (Fig. 3, lane A) exhibited 2.6- and 2.8-kb *Pvu*II fragments which bound this *uspA1*-derived probe. In contrast, the mutant strain (Fig. 3, lane G) had 2.6- and 3.4-kb *Pvu*II fragments that bound this probe. The presence of the 3.4-kb *Pvu*II fragment was the result of allelic exchange involving the mutated *uspA1* gene containing the *kan* cartridge; only the 3.4-kb *Pvu*II fragment of the mutant bound a *kan* cartridge probe (data not shown). Therefore, we inferred from this result that the 2.8-kb *Pvu*II fragment from strain 035E (Fig. 3, lane A) contained the *uspA1* ORF.

TABLE 2. Amino acid sequence of peptides derived from the SDS-PAGE-purified, very-high-molecular-weight UspA protein from *M. catarrhalis* 035E

Type of peptides	Sequences
Similar or identical[a]	KALESNVEEGLLDLSGR
	ALESNVEEGLLELSGRTIDQR
	NQAHIANNINXIYELAQQQDQK[c]
	NQADIAQNQTDIQDLAAYNELQ
	ATHDYNERQTEA
	KASSENTQNIAK
Other[b]	MILGDTAIVSNSQDNKTQLKFYK
	AGDTIIPLDDDXXP
	LLHEQQLXGK
	IFFNXG

[a] Peptides identical or very similar to peptides contained in the deduced amino acid sequence of the UspA1 protein.

[b] Peptides which match poorly or not at all with the deduced amino acid sequence of the UspA1 protein.

[c] X indicates a residue that was not identified.

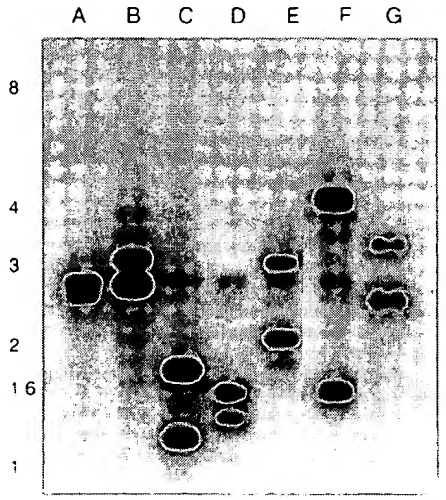


FIG. 3. Southern blot analysis of *Pvu*II-digested chromosomal DNA from wild-type and mutant strains of *M. catarrhalis* with a probe containing *uspA1* DNA. The 600-bp *Bgl*II-*Pvu*II fragment from pJL501.6 was used as the probe. Lanes: A, 035E; B, TTA24; C, TTA1; D, TTA37; E, P44; F, ATCC 25240; G, *uspA1* mutant strain 035E.1. Kilobase position markers are indicated to the left of the figure.

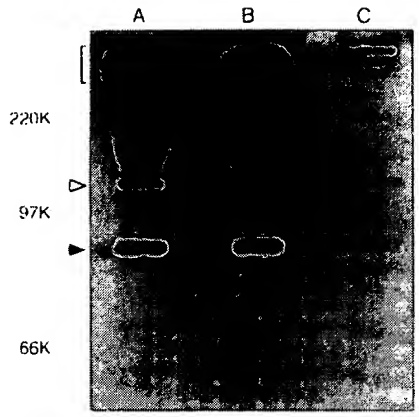


FIG. 4. Western blot analysis of outer membrane proteins in wild-type and mutant *M. catarrhalis* strains by use of autoradiography. Proteins present in EDTA-extracted outer membrane vesicles from the wild-type strain 035E (lane A), the isogenic *uspA1* mutant strain 035E.1 (lane B), and the isogenic *uspA2* mutant strain 035E.2 (lane C) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with MAb 17C7 followed by radioiodinated goat anti-mouse immunoglobulin. The bracket on the left indicates the very-high-molecular-weight form of the UspA protein. The open arrowhead indicates the 120-kDa, putative monomeric form of the UspA1 protein. The closed arrowhead indicates the 85-kDa, putative monomeric form of the UspA2 protein. This autoradiogram had to be overexposed to detect the 120- and 85-kDa proteins; shorter exposure times revealed that the very-high-molecular-weight form of the UspA protein expressed by both the wild-type strain and the *uspA1* mutant formed a discrete band that bound MAb 17C7 and also gave rise to a number of apparent degradation products which migrated slightly faster in SDS-PAGE. Molecular weight position markers (K, thousand) are shown to the left of the figure.

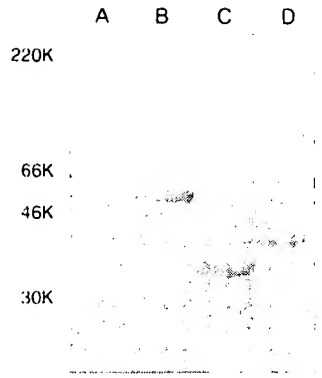


FIG. 5. Western blot analysis of the reactivity of UspA1-derived fusion proteins with MAb 17C7. The MAb 17C7-reactive fusion proteins MF-4 (lane B), MF-4-1 (lane C), and MF-4-2 (lane D) were produced by plasmid constructs generated as described in Results. Fusion protein MF-3 (lane A) contains amino acid residues 289 to 474 from UspA1; the plasmid construct expressing this fusion protein was produced by use of oligonucleotide primers P5 and P8 in Fig. 2 for PCR; it is included here as a negative control. Molecular weight position markers (K, thousand) are shown to the left of the figure.

Identification of MAb 17C7-reactive proteins. Western blot analysis of outer membrane vesicles of the *uspA1* mutant strain 035E.1 (Fig. 4, lane B) revealed that this mutant still expressed the very-high-molecular-weight protein reactive with MAb 17C7 and at a level equivalent to that expressed by the wild-type strain (Fig. 4, lane A). However, long-term exposure of this autoradiogram revealed that the *uspA1* mutant (Fig. 4, lane B) lacked expression of an antigen with an apparent molecular weight of approximately 120,000 that was expressed by the wild-type strain (Fig. 4, lane A).

The fact that this *uspA1* mutant still expressed the very-high-molecular-weight UspA antigen reactive with MAb 17C7 indicated that there had to be a second gene in *M. catarrhalis* 035E that encoded a MAb 17C7-reactive protein. In this context, it should be noted that both the wild-type strain and the *uspA1* mutant (Fig. 4, lanes A and B, respectively) expressed an antigen with an apparent molecular weight of approximately 85,000 that bound MAb 17C7. Identification of the gene encoding this 85-kDa protein was successfully accomplished by use of a combination of epitope mapping and PCR methods as described below.

Localization of the UspA1 epitope that binds MAb 17C7. The UspA1 epitope which bound MAb 17C7 was localized by use of the nucleotide sequence of the *uspA1* gene to construct GST-fusion proteins. The epitope that bound MAb 17C7 was localized first to a 222-amino-acid span (encoded by nucleotides 1638 to 2303 [Fig. 2]) contained in the fusion protein MF-4 (Fig. 5, lane B). The oligonucleotide primers (P6 and P13) used in the PCR to amplify the relevant nucleotide sequence from *M. catarrhalis* 035E chromosomal DNA are depicted in Fig. 2. Further analysis of the UspA1-derived amino acid sequence in the MF-4 fusion construct involved the production of fusion proteins containing 78 amino acid residues (MF-4-1, derived from the use of primers P7 and P12 [Fig. 2]) and 123 amino acid residues (MF-4-2, derived from the use of primers P11 and P13). Both MF-4-1 and MF-4-2 bound MAb 17C7 (Fig. 5, lanes C and D, respectively) and had in common a 23-residue region, suggesting, although not proving, that this 23-residue region contained the epitope that bound MAb 17C7.

Identification of a second gene in *M. catarrhalis* 035E encoding the MAb 17C7-reactive epitope. It is important to note that the nucleotide sequence encoding the 23-amino-acid region common to both MF-4-1 and MF-4-2 was present in the 600-bp *Bgl*II-*Pvu*II fragment (Fig. 2) used in the Southern blot analysis described above (Fig. 3). This finding suggested that the epitope that bound MAb 17C7 might be encoded by DNA present in both the 2.6- and 2.8-kb *Pvu*II fragments from *M. catarrhalis* 035E that hybridized with this probe (Fig. 3, lane A). Moreover, by comparison with the Southern blot results obtained with the isogenic *uspA1* mutant (Fig. 3, lane G), it was apparent that the wild-type 2.8-kb *Pvu*II fragment (Fig. 3, lane A) contained *uspA1* DNA and that the wild-type 2.6-kb *Pvu*II fragment (Fig. 3, lane A) likely represented all or part of another gene encoding this same epitope.

This hypothesis was tested by means of the previously described ligation-based PCR system. Chromosomal DNA from the isogenic *uspA1* mutant was digested to completion with *Pvu*II and resolved by agarose gel electrophoresis. Fragments ranging in size from 2 to 3 kb were excised from the agarose, blunt ended, and ligated into the *Eco*RV site in pBS. This ligation reaction mixture was precipitated and used in a PCR amplification. Each PCR contained an oligonucleotide primer for the vector together with an oligonucleotide primer (either P9 or P10 [Fig. 2]) derived from the DNA near the center of the MF-4 insert. This approach yielded a 1.7- to 1.8-kb product with the vector and P10 primers and a 0.8- to 0.9-kb product with the vector and P9 primers. It should be noted that the sum of the sizes of these two bands is approximately the same as the 2.6-kb size of the desired DNA fragment.

Nucleotide sequence analysis of these two PCR products revealed the presence of a partial ORF in each; one contained a putative translation initiation codon, and the other contained a termination codon. When joined at the region containing the P9 and P10 primers (Fig. 6), these two partial ORFs formed a

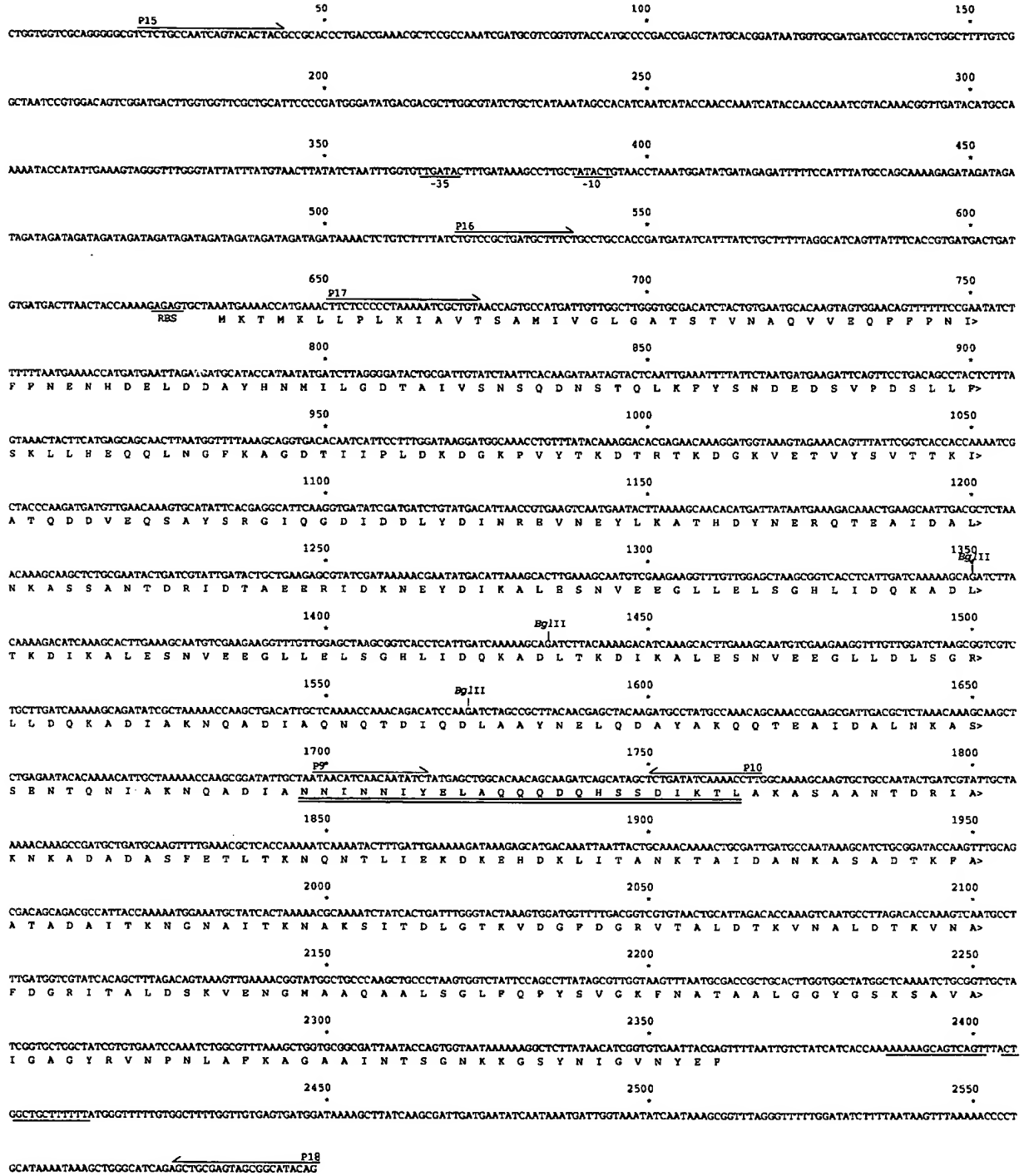


FIG. 6. Nucleotide sequence of the *uspA2* gene from *M. catarrhalis* 035E and the deduced amino acid sequence of the UspA2 protein. Putative −35, −10, and ribosome binding sites (RBS) are indicated. Fifteen repeats of the tetranucleotide AGAT are present between nucleotides 439 and 499. Opposing arrows indicate an inverted repeat immediately downstream from the ORF. Oligonucleotide primers (P15 to P18) used for PCR-based amplification of selected regions of this sequence from the chromosome of the wild-type *M. catarrhalis* strain 035E are indicated by arrows placed above the relevant sequences. Primers P9 and P10 are included here to indicate the *uspA2* DNA regions that bound these primers in the ligation-based PCR experiment. The *Bgl*II sites used for insertion of the *kan* cartridge are indicated; the peptide that is also present in both MF-4-1 and MF-4-2 is double underlined.

complete 1.7-kb ORF (Fig. 6). Oligonucleotide primers P16 and P18 (Fig. 6) were used to amplify a 2.6-kb fragment from *M. catarrhalis* 035E chromosomal DNA. Nucleotide sequence analysis of this PCR product was used to confirm the nucleotide sequence of the ORF, designated *uspA2*, determined from the ligation-based PCR experiment. When this same PCR product was ligated into the ZAP Express bacteriophage, the resultant recombinant bacteriophage formed plaques which bound MAb 17C7 (data not shown), thus confirming that the *uspA2* ORF encoded a MAb 17C7-reactive protein.

Features of the *uspA2* gene and its encoded protein product. The *uspA2* ORF contained 1,731 nucleotides encoding a protein containing 576 amino acids with a calculated molecular weight of 62,483. Putative −10, −35, and ribosome binding sites are indicated in Fig. 6. Interestingly, there were 15 repeats of the tetranucleotide AGAT located 150 nucleotides 5′ from the predicted translation initiation codon (Fig. 6); the significance of these repeats is not known. An inverted repeat was located immediately downstream from this ORF (nucleotides 2381 to 2412 [Fig. 6]). Immediately upstream from the *uspA2* gene was a gene encoding a product that is most similar to the glycoprotease of *Pasteurella haemolytica* (1). A gene encoding a predicted protein that resembled most closely the MetR regulatory protein of *E. coli* (43) was located downstream from *uspA2* and on the opposite strand. Similar to UspA1, UspA2 contained several different amino acid repeat motifs with two possible leucine zippers (data not shown). When the amino acid sequence of UspA2 was used in a BLAST search of GenBank, the UspA2 protein proved to be most similar (i.e., 27% identical and 47% similar by GAP alignment) to the YadA outer membrane protein of pathogenic *Yersinia* species (59).

The amino acid sequence of UspA2 was 43% identical to that of UspA1. However, closer examination revealed that a region containing amino acids 271 to 411 in UspA2 was 93% identical to the region containing amino acids 498 to 638 in UspA1 (Fig. 7). Outside of these regions, the level of identity was only 22 to 24%. It also should be noted that the four previously described peptides that were not found in UspA1 (Table 2) were found to be very similar to peptides in the deduced amino acid sequence of UspA2. The degree of identity between these four peptides and their counterparts in UspA2 ranged from 67 to 91% (ClustalW score range, 46 to 138). In addition, the peptides which matched or were very similar to peptides in the deduced amino acid sequence of UspA1 (Table 2) also matched peptides found in the deduced amino acid sequence of UspA2.

Construction and analysis of a *uspA2* mutant. To confirm that the 62-kDa UspA2 protein could form the high-molecular-weight UspA antigen, an isogenic *uspA2* mutant was constructed. The oligonucleotide primers P17 and P18 (Fig. 6) were used to amplify a 2-kb product from the *M. catarrhalis* 035E chromosome; this fragment lacked 15 bp from the 5′ end of the *uspA2* ORF. This PCR product was cloned into pBS, yielding the recombinant plasmid pUSPA2. This construct was digested with *Bgl*II, which cut the *uspA2* ORF three times within a 230-bp region (Fig. 6); a *kan* cartridge was inserted into this deletion site, yielding pUSPA2KAN. This mutated DNA was used to electroporate the wild-type strain 035E, and a kanamycin-resistant transformant, designated 035E.2, was selected at random. PCR-based analysis, using oligonucleotide primers P17 and P18 (Fig. 6), indicated that allelic exchange had occurred in strain 035E.2, with the mutated *uspA2* gene replacing the wild-type allele (data not shown).

Western blot analysis with MAb 17C7 determined that outer membrane vesicles of the *uspA2* mutant strain (Fig. 4, lane C) lacked expression of the 85-kDa antigen detected previously in both the wild-type strain and the isogenic *uspA1* mutant (Fig. 4, lanes A and B, respectively). In addition, the very-high-molecular-weight form of the MAb 17C7-reactive antigen was greatly reduced in apparent abundance in outer membrane vesicles from the isogenic *uspA2* mutant (Fig. 4, lane C). The 120-kDa antigen that was missing from the *uspA1* mutant (Fig. 4, lane B) was expressed by the *uspA2* mutant (Fig. 4, lane C).

Southern blot analysis of other *M. catarrhalis* strains by use of *uspA1*- and *uspA2*-specific probes. Chromosomal DNA extracted from *M. catarrhalis* 035E, from four additional disease isolates of this organism, and from *M. catarrhalis* ATCC 25240 was digested with *Pvu*II and used in Southern blot analysis with *uspA1*- and *uspA2*-specific DNA probes. For each strain, the *Pvu*II fragment that bound the *uspA1*-specific probe was dif-

```
UspA1  101 YSTIVGGGYNKAEGRYSTIGGGSNNEATNEYSTIVGGDDNKATGRYSTIG 150
UspA2    1 ..............................MKTMKLLPLKIAVTSAMIVG  20

       151 GGDNNTREGEYSTVAGGKNNQATGTGSFAAGVENQANAENAVAVGKKNII 200
        21 LGAT.......STVNAQVVEQFFPNIFFNENHDELDDAYHNMILGDTAIV   6

       201 EGENSVAIGSENTVKTEHKNVFILGSGTTGVTSNSVLLGNETAGKQATTV 250
        64 SNSQ.............DNSTQLKFYSNDEDSVPDSLLFSKLLHEQQ....  97

       251 KNAEVGGLSLTGFAGESKAENGVVSVGSEGGERQIVNVGAGQISDTSTDA 300
        98 .........LNGF....KAGDTIIPLDKDG.........KPVYTKDTRTKD 126

       301 VNGSQLHALATVVDDNQYDIVNNRADILNNQDDIKDLQKEVKGLDNEVGE 350
       127 GKVETVYYSVTTKIATQ..DDVEQSAYSRGIQGDIDDL....YDINREVNE 170

       351 LSRDINSLHDVTDNQQDDIKELKRGVKELDNEVGVLSRDINSLHDDVADN 400
       171 Y...LKATHDYNERQTEAIDALNKASSANT..............DRIDTA 203

       401 QDDIAKNKADIKGLNKEVKELDKEVGVLSRDIGSLHDDVATNQADIAKNQ 450
       204 EERIDKNEYDIKALESNVEE..........GLLELSGHLIDQKADLTK..  241

       451 ADIKTLENNVEEELLNLSGRLLDQKADIDNNINNIYELAQQQDQHSSDIK 500
       242 .DIKALESNVEEGLLELSGHLIDQKADLTK.................DIK 273

       501 TLKNNVEEGLLDLSGRLIDQKADIAKNQADIAQNQTDIQDLAAYNELQDQ 550
       274 ALESNVEEGLLDLSGRLLDQKADIAKNQADIAQNQTDIQDLAAYNELQDA 323

       551 YAQKQTEAIDALNKASSENTQNIAKNQADIANNINNIYELAQQQDQHSSD 600
       324 YAKQQTEAIDALNKASSENTQNIAKNQADIANNINNIYELAQQQDQHSSD 373

       601 IKTLAKVSAANTDRIAKNKAEADASFETLTKNQNTLIEQGEALVEQNKAI 650
       374 IKTLAKASAANTDRIAKNKADADASFETLTKNQNTLIEKDKE........ 415

       651 NQSLEGFAAHADIQDKQILQNQADITTNKTAIEQNINRTVANGFEIEKNK 700
       416 .............HDKLITANKTAIDANKASADTKFAATAD...AITKNG 449

       701 AGIATNKQELILQNDRLNRINETNNRQDQKIDQLGYALKEQGQHFNNRIS 750
       450 NAITKNAKSITDLGTKVDGFDGRVTALDTKVN....ALDTKVNAFDGRIT 495

       751 AVERQTAGGIANAIAIATLPSPSRAGEHHVLFGSGYHNGQAAVSLGAAGL 800
       496 ALDSKVENGMAAQAALSGLFQPYSVGKFNATAALGGYGSKSAVAIG.AGY 544

       801 SDTGKSTYKIGLSWSDAGGLSGG..VGGSYRWK 831
       545 RVNPNLAFKAGAAINTSGNKKGSYNIGVNYEF. 576
```

FIG. 7. Comparison of the deduced amino acid sequences of the UspA1 and UspA2 proteins from *M. catarrhalis* 035E as determined by use of the GAP alignment program in the University of Wisconsin sequence analysis package. The first 100 residues of the UspA1 protein are not included. Vertical bars indicate identity, colons indicate conserved substitutions, and single dots indicate less-conserved substitutions.

ferent from the *Pvu*II fragment that bound the *uspA2*-specific probe (data not shown).

DISCUSSION

Previous studies from both this laboratory (27) and another (34) described a very-high-molecular-weight protein of *M. catarrhalis* that is expressed by all *M. catarrhalis* disease isolates examined to date. This protein, designated UspA (27) or HMWP (34), has also been shown to be a target for a MAb (17C7) which enhanced pulmonary clearance of this organism in a murine model system and bound to all strains of *M. catarrhalis* tested to date (27). However, the findings of the current study indicate that there are two genes in *M. catarrhalis* 035E that encode the epitope that binds MAb 17C7.

From the available mutant strain analysis data, it is clear that the *uspA1* and *uspA2* gene products can be detected as 120- and 85-kDa proteins, respectively, in Western blot analysis (Fig. 4). These two MAb 17C7-reactive antigens likely represent the monomeric forms of the UspA1 and UspA2 proteins, respectively. At this time, we do not know why these two proteins migrate more slowly in SDS-PAGE (i.e., they have apparent molecular weights of 120,000 and 85,000) than would be expected from their calculated molecular weights (i.e., 88,271 and 62,483). It is interesting to note that the calculated molecular weights for both UspA1 and UspA2 are 73% of the apparent molecular weights of their putative monomeric forms as estimated by SDS-PAGE.

It is also now apparent that both UspA1 and UspA2 form aggregates or oligomers that migrate with an estimated molecular weight of at least 250,000 in SDS-PAGE (Fig. 4). The need to overexpose the autoradiogram to detect the 120- and 85 kDa proteins (Fig. 4) suggested that relatively few of these putative monomeric forms are present in *M. catarrhalis* cells. In addition, it would also appear that the UspA2 protein likely comprises the bulk of the protein present in the very-high-molecular-weight form of the UspA protein in strain 035E (Fig. 4, compare lanes B and C).

The fact that MAb 17C7 binds to the surface of whole *M. catarrhalis* cells (27) indicates that the homologous epitope is exposed to the environment. Whether both UspA1 and UspA2 are exposed on the surface of *M. catarrhalis* cannot be determined from the available data. The deduced amino acid sequence of UspA2 contained what could be a leader peptide, with a hydrophobic region of approximately 20 amino acids at the N terminus that was preceded by one or two basic amino acids (Fig. 6). In contrast, with the assumption that the translation initiation codon depicted in Fig. 2 is correct, the N-terminal region of UspA1 (Fig. 2) did not resemble a leader peptide. At this time, we cannot formally exclude the possibility that the UspA1 protein detected in Western blot analysis of outer membrane vesicles (Fig. 4) is primarily localized elsewhere in the *M. catarrhalis* cell. Conclusive determination of which of these proteins is surface exposed will necessarily have to await development of polyclonal antibody or MAb probes specific for each protein.

The apparent molecular weight of UspA in SDS-PAGE has been reported previously as ranging from 300,000 to 700,000 (27, 34). Treatment of either purified UspA or *M. catarrhalis* outer membrane vesicles with reducing agents, alkylating agents, or heat failed to alter the migration characteristics of this macromolecule in SDS-PAGE (27, 34). However, formic acid treatment of purified, very-high-molecular-weight UspA prior to SDS-PAGE was reported to result in the appearance of a single band with an apparent molecular weight of between 120,000 and 140,000 (34). The 120-kDa antigen expressed by the *uspA1* gene in the present study (Fig. 4) is very similar in size to this product obtained from the formic acid-treated UspA (34), suggesting that UspA1 may have been selectively purified in this previous study. Alternatively, the *M. catarrhalis* strain used in this previous study (34) may have expressed only or mostly UspA1.

The UspA1 protein resembled most closely a surface fibril-forming macromolecule from *H. influenzae* type b that has been implicated in the ability of this encapsulated organism to attach to epithelial cells (60, 61). Genetic analysis of an isogenic *hsf* mutant of *H. influenzae* type b indicated that this mutant lost essentially all of its ability to adhere to Chang conjunctival cells in vitro (60). Similarly, a recombinant *E. coli* strain that expressed the *hsf* gene product exhibited a greatly increased ability to adhere to these same epithelial cells (61). Whether UspA1 can form fibrils on the surface of *M. catarrhalis* or plays some role in the ability of *M. catarrhalis* to colonize the nasopharynx cannot be determined from the available data.

The *M. catarrhalis* UspA2 protein resembled a virulence factor of another pathogen, being most similar to the YadA adhesin-invasin expressed by pathogenic *Yersinia* species. YadA has been implicated in numerous virulence-associated phenotypes, including serum resistance of *Yersinia enterocolitica* (8, 9, 50), adherence to and entry into HEp-2 cells by *Yersinia pseudotuberculosis* (4), binding of *Yersinia* organisms to extracellular matrix components including fibronectin and/or collagen (54, 55, 63, 64), and formation of surface tendrils (fibrillae) (31, 39, 69). Of particular interest, with respect to its similarity to UspA2, is the fact that the native YadA antigen migrates with an apparent molecular weight of approximately 200,000 in SDS-PAGE (58, 69). This 200-kDa antigen actually represents an oligomer comprised of several 45- to 50-kDa YadA monomers (40).

At the level of primary structure, UspA1 and UspA2 are not very similar to each other except in one specific region. There is only 22% identity between UspA1 residues 1 to 450 and UspA2 residues 1 to 240 and just 24% identity between UspA1 residues 649 to 831 and UspA2 residues 415 to 576. In contrast, there is 93% identity between UspA1 residues 498 to 638 and UspA2 residues 271 to 411 (Fig. 7). Furthermore, these two regions both contain the 23-residue peptide that may contain the epitope that binds MAb 17C7 (Fig. 2 and 6), with this peptide being repeated once in UspA1 (Fig. 2). The genetic basis for this apparent duplication of DNA internal to both genes is not known at this time.

The available data indicate that *M. catarrhalis* 035E expressed both UspA1 and UspA2. Southern blot analysis using probes specific for *uspA1* and *uspA2* revealed that other disease isolates of *M. catarrhalis* each possessed two distinct chromosomal DNA fragments that hybridize with these probes (data not shown). This latter result suggests that most disease isolates of this pathogen have the potential to express both UspA1 and UspA2. Determination of whether all strains of *M. catarrhalis* simultaneously express both of these macromolecules will require the development of the UspA1- and UspA2-specific antibody probes mentioned above.

The likelihood that *M. catarrhalis* expresses both of these proteins necessitates reevaluation of published reports concerning the antigenic and immunogenic properties of the UspA protein. Antibodies to UspA have been demonstrated to be present in convalescent-phase sera from patients with documented *M. catarrhalis* pneumonia (27), providing evidence that UspA is expressed by *M. catarrhalis* growing in vivo. It has also been suggested that UspA may play a role in the resistance of some *M. catarrhalis* isolates to killing by normal human

serum (66). Most recently, it was shown that immunization with purified UspA resulted in enhanced pulmonary clearance of homologous and heterologous *M. catarrhalis* strains from the lungs of mice (7). All of these previous studies need to be reinterpreted with respect to the identity of the antigen or immunogen (i.e., UspA1, UspA2, or both) being examined.

The functions of UspA1 and UspA2 in *M. catarrhalis* remain to be defined. It is noteworthy that both UspA1 and UspA2 resemble prokaryotic adhesins, and the possibility that these macromolecules are somehow involved in the adherence of *M. catarrhalis* to human nasopharyngeal mucosa is particularly intriguing. It also has been suggested, in a preliminary report, that the UspA protein may be involved in binding of vitronectin and in complement resistance (66). Direct investigation of the possible involvement of the UspA1 and UspA2 proteins in the interaction between *M. catarrhalis* and components of both the human respiratory tract and the complement system should now be feasible.

ACKNOWLEDGMENTS

This study was supported by U.S. Public Health Service grant AI36344 and by Texas Advanced Technology Program award 003660-087 to E.J.H.

We thank Steven Berk for providing several of the *M. catarrhalis* disease isolates used in this study.

REFERENCES

1. **Abdullah, K. M., R. Y. C. Lo, and A. Mellors.** 1991. Cloning, nucleotide sequence, and expression of the *Pasteurella haemolytica* A1 glycoprotease gene. J. Bacteriol. **173**:5597–5603.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403–410.
3. **Bartos, L. C., and T. F. Murphy.** 1988. Comparison of the outer membrane proteins of 50 strains of *Branhamella catarrhalis*. J. Infect. Dis. **158**:761–765.
4. **Bliska, J. B., M. C. Copass, and S. Falkow.** 1993. The *Yersinia pseudotuberculosis* adhesin YadA mediates intimate bacterial attachment to and entry into HEp-2 cells. Infect. Immun. **61**:3914–3921.
5. **Bluestone, C. D.** 1986. Otitis media and sinusitis in children: role of *Branhamella catarrhalis*. Drugs **31**(Suppl. 3):132–141.
6. **Catlin, B. W.** 1990. *Branhamella catarrhalis*: an organism gaining respect as a pathogen. Clin. Microbiol. Rev. **3**:293–320.
7. **Chen, D., J. C. McMichael, K. R. van der Meid, D. Hahn, T. Mininni, J. Cowell, and J. Eldridge.** 1996. Evaluation of purified UspA from *Moraxella catarrhalis* as a vaccine in a murine model after active immunization. Infect. Immun. **64**:1900–1905.
8. **China, B., B. T. N'Guyen, M. de Bruyere, and G. R. Cornelis.** 1994. Role of YadA in resistance of *Yersinia enterocolitica* to phagocytosis by human polymorphonuclear leukocytes. Infect. Immun. **62**:1275–1281.
9. **China, B., M.-P. Sory, B. T. N'Guyen, M. de Bruyere, and G. R. Cornelis.** 1993. Role of YadA protein in prevention of opsonization of *Yersinia enterocolitica* by C3b molecules. Infect. Immun. **61**:3129–3136.
10. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
11. **Faden, H.** 1995. Comparison of the local immune response to nontypeable *Haemophilus influenzae* (nHI) and *Moraxella catarrhalis* (MC) during otitis media, p. 733–736. *In* J. Mestecky et al. (ed.), Advances in mucosal immunology. Plenum Press, New York, N.Y.
12. **Faden, H., Y. Harabuchi, J. J. Hong, and Tonawanda/Williamsburg Pediatrics.** 1994. Epidemiology of *Moraxella catarrhalis* in children during the first 2 years of life: relationship to otitis media. J. Infect. Dis. **169**:1312–1317.
13. **Faden, H., J. J. Hong, and N. Pahade.** 1994. Immune response to *Moraxella catarrhalis* in children with otitis media: opsonophagocytosis with antigen-coated latex beads. Ann. Otol. Rhinol. Laryngol. **103**:522–524.
14. **Faden, H. S., J. J. Hong, and T. F. Murphy.** 1992. Immune response to outer membrane antigens of *Moraxella catarrhalis* in children with otitis media. Infect. Immun. **60**:3824–3829.
15. **Giebink, G. S.** 1989. The microbiology of otitis media. Pediatr. Infect. Dis. J. **8**:S18–S20.
16. **Gish, W., and D. J. States.** 1993. Identification of protein coding regions by database similarity search. Nat. Genet. **3**:266–272.
17. **Goldblatt, D., G. K. Scadding, V. J. Lund, A. M. Wade, M. W. Turner, and J. P. Pandey.** 1994. Association of Gm allotypes with the antibody response to the outer membrane proteins of a common upper respiratory tract organism, *Moraxella catarrhalis*. J. Immunol. **153**:5316–5320.
18. **Goldblatt, D., N. D. Seymour, R. J. Levinsky, and M. W. Turner.** 1990. An enzyme-linked immunosorbent assay for the determination of human IgG subclass antibodies directed against *Branhamella catarrhalis*. J. Immunol. Methods **128**:219–225.
19. **Goldblatt, D., M. W. Turner, and R. J. Levinsky.** 1990. *Branhamella catarrhalis*: antigenic determinants and the development of the IgG subclass response in childhood. J. Infect. Dis. **162**:1128–1135.
20. **Gray-Owen, S. D., S. Loosmore, and A. B. Schryvers.** 1995. Identification and characterization of genes encoding the human transferrin-binding proteins from *Haemophilus influenzae*. Infect. Immun. **63**:1201–1210.
21. **Gulig, P. A., G. H. McCracken, Jr., C. F. Fritsch, K. H. Johnston, and E. J. Hansen.** 1982. Antibody response of infants to cell surface-exposed outer membrane proteins of *Haemophilus influenzae* type b after systemic *Haemophilus* disease. Infect. Immun. **37**:82–88.
22. **Gulig, P. A., C. C. Patrick, L. Hermanstorfer, G. H. McCracken, Jr., and E. J. Hansen.** 1987. Conservation of epitopes in the oligosaccharide portion of the lipooligosaccharide of *Haemophilus influenzae* type b. Infect. Immun. **55**:513–520.
23. **Hager, H., A. Verghese, S. Alvarez, and S. L. Berk.** 1987. *Branhamella catarrhalis* respiratory infections. Rev. Infect. Dis. **9**:1140–1149.
24. **Hansen, E. J., J. L. Latimer, S. E. Thomas, M. E. Helminen, W. L. Albritton, and J. D. Radolf.** 1992. Use of electroporation to construct isogenic mutants of *Haemophilus ducreyi*. J. Bacteriol. **174**:5442–5449.
25. **Hansen, E. J., S. E. Pelzel, K. Orth, C. R. Moomaw, J. D. Radolf, and C. A. Slaughter.** 1989. Structural and antigenic conservation of the P2 porin protein among strains of *Haemophilus influenzae* type b. Infect. Immun. **57**: 3270–3275.
26. **Helminen, M. E., I. Maciver, J. L. Latimer, L. D. Cope, G. H. McCracken, Jr., and E. J. Hansen.** 1993. A major outer membrane protein of *Moraxella catarrhalis* is a target for antibodies that enhance pulmonary clearance of the pathogen in an animal model. Infect. Immun. **61**:2003–2010.
27. **Helminen, M. E., I. Maciver, J. L. Latimer, J. Klesney-Tait, L. D. Cope, M. M. Paris, G. H. McCracken, Jr., and E. J. Hansen.** 1994. A large, antigenically conserved protein on the surface of *Moraxella catarrhalis* is a target for protective antibodies. J. Infect. Dis. **170**:867–872.
28. **Helminen, M. E., I. Maciver, J. L. Latimer, S. R. Lumbley, L. D. Cope, G. H. McCracken, Jr., and E. J. Hansen.** 1993. A mutation affecting expression of a major outer membrane protein of *Moraxella catarrhalis* alters serum resistance and survival of this organism in vivo. J. Infect. Dis. **168**:1194–1201.
29. **Hol, C., C. M. Verduin, E. E. A. Van Dijke, J. Verhoef, A. Fleer, and H. van Dijk.** 1995. Complement resistance is a virulence factor of *Branhamella* (*Moraxella*) *catarrhalis*. FEMS Immunol. Med. Microbiol. **11**:207–212.
30. **Hsiao, C. B., S. Sethi, and T. F. Murphy.** 1995. Outer membrane protein CD of *Branhamella catarrhalis*—sequence conservation in strains recovered from the human respiratory tract. Microb. Pathog. **19**:215–225.
31. **Kapperud, G., E. Namork, M. Skurnik, and T. Nesbakken.** 1987. Plasmid-mediated surface fibrillae of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*: relationship to the outer membrane protein YOP1 and possible importance for pathogenesis. Infect. Immun. **55**:2247–2254.
32. **Kimura, A., P. A. Gulig, G. H. McCracken, Jr., T. A. Loftus, and E. J. Hansen.** 1985. A minor high-molecular-weight outer membrane protein of *Haemophilus influenzae* type b is a protective antigen. Infect. Immun. **47**: 253–259.
33. **Klesney-Tait, J., T. J. Hiltke, S. M. Spinola, J. D. Radolf, and E. J. Hansen.** 1997. The major outer membrane protein of *Haemophilus ducreyi* consists of two OmpA homologs. J. Bacteriol. **179**:1764–1773.
34. **Klingman, K. L., and T. F. Murphy.** 1994. Purification and characterization of a high-molecular-weight outer membrane protein of *Moraxella* (*Branhamella*) *catarrhalis*. Infect. Immun. **62**:1150–1155.
35. **Klingman, K. L., A. Pye, T. F. Murphy, and S. L. Hill.** 1995. Dynamics of respiratory tract colonization by *Branhamella catarrhalis* in bronchiectasis. Am. J. Respir. Crit. Care Med. **152**:1072–1078.
36. **Kovatch, A. L., E. R. Wald, and R. H. Michaels.** 1983. Beta-lactamase-producing *Branhamella catarrhalis* causing otitis media in children. J. Pediatr. **102**:261–264.
37. **Kroll, J. S., P. R. Langford, J. R. Saah, and B. M. Loynds.** 1993. Molecular and genetic characterization of superoxide dismutase in *Haemophilus influenzae* type b. Mol. Microbiol. **10**:839–848.
38. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.
39. **Lachica, R. V., D. L. Zink, and W. R. Ferris.** 1984. Association of fibril structure formation with cell surface properties of *Yersinia enterocolitica*. Infect. Immun. **46**:272–275.
40. **Mack, D. H., J. Heesemann, and R. Laufs.** 1994. Characterization of different oligomeric species of the *Yersinia enterocolitica* outer membrane protein YadA. Med. Microbiol. Immunol. **183**:217–227.
41. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
42. **Matsudaira, P.** 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262**: 10035–10038.
43. **Maxon, M. E., J. Wigboldus, N. Brot, and H. Weissbach.** 1990. Structure-

function studies on *Escherichia coli* MetR protein, a putative prokaryotic leucine zipper protein. Proc. Natl. Acad. Sci. USA 87:7076–7079.
44. **McLeod, D. T., F. Ahmad, S. Capewell, M. J. Croughan, M. A. Calder, and A. Seaton.** 1986. Increase in bronchopulmonary infection due to *Branhamella catarrhalis*. Br. Med. J. Clin. Res. 292:1103–1105.
45. **Moxon, E. R., and R. Wilson.** 1991. The role of *Haemophilus influenzae* in the pathogenesis of pneumonia. Rev. Infect. Dis. 13(Suppl. 6):S518–S527.
46. **Murphy, T. F., and L. C. Bartos.** 1989. Surface-exposed and antigenically conserved determinants of outer membrane proteins of *Branhamella catarrhalis*. Infect. Immun. 57:2938–2941.
47. **Murphy, T. F., C. Kirkham, and A. J. Lesse.** 1993. The major heat-modifiable outer membrane protein CD is highly conserved among strains of *Branhamella catarrhalis*. Mol. Microbiol. 10:87–97.
48. **Murphy, T. F., and M. R. Loeb.** 1989. Isolation of the outer membrane of *Branhamella catarrhalis*. Microb. Pathog. 6:159–174.
49. **Nicotra, B., M. Rivera, J. I. Liman, and R. J. Wallace.** 1986. *Branhamella catarrhalis* as a lower respiratory tract pathogen in patients with chronic lung disease. Arch. Intern. Med. 146:890–893.
50. **Pilz, D., T. Vocke, J. Heesemann, and V. Brade.** 1992. Mechanism of YadA-mediated serum resistance of *Yersinia enterocolitica* serotype O3. Infect. Immun. 60:189–195.
51. **Post, J. C., R. A. Preston, J. J. Aul, M. Larkins-Pettigrew, J. Rydquist-White, K. W. Anderson, R. M. Wadowsky, D. R. Reagan, E. S. Walker, L. A. Kingsley, A. E. Magit, and G. D. Ehrlich.** 1995. Molecular analysis of bacterial pathogens in otitis media with effusion. JAMA 273:1598–1604.
52. **Postle, K., and R. F. Good.** 1985. A bidirectional rho-independent transcription terminator between the *E. coli tonB* gene and an opposing gene. Cell 41:577–585.
53. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
54. **Schulze-Koops, H., H. Burkhardt, J. Heesemann, T. Kirsch, B. Swoboda, C. Bull, S. Goodman, and F. Emmrich.** 1993. Outer membrane protein YadA of enteropathogenic yersiniae mediates specific binding to cellular but not plasma fibronectin. Infect. Immun. 61:2513–2519.
55. **Schulze-Koops, H., H. Burkhardt, J. Heesemann, K. von der Mark, and F. Emmrich.** 1992. Plasmid-encoded outer membrane protein YadA mediates specific binding of enteropathogenic yersiniae to various types of collagen. Infect. Immun. 60:2153–2159.
56. **Shine, J., and L. Dalgarno.** 1975. Determinant of cistron specificity in bacterial ribosomes. Nature 254:34–38.
57. **Shurin, P. A., C. D. Marchant, C. H. Kim, G. F. van Hare, C. E. Johnson, M. A. Tutihasi, and L. J. Knapp.** 1983. Emergence of beta-lactamase-producing strains of *Branhamella catarrhalis* as important agents of acute otitis media. Pediatr. Infect. Dis. J. 2:34–38.
58. **Skurnik, M., I. Bolin, H. Heikkinen, S. Piha, and H. Wolf-Watz.** 1984. Virulence plasmid-associated autoagglutination in *Yersinia* spp. J. Bacteriol. 158:1033–1036.
59. **Skurnik, M., and H. Wolf-Watz.** 1989. Analysis of the *yopA* gene encoding the Yop1 virulence determinants of *Yersinia* spp. Mol. Microbiol. 3:517–529.
60. **St. Geme, J. W., III, and D. Cutter.** 1995. Evidence that surface fibrils expressed by *Haemophilus influenzae* type b promote attachment to human epithelial cells. Mol. Microbiol. 15:77–85.
61. **St. Geme, J. W., III, D. Cutter, and S. J. Barenkamp.** 1996. Characterization of the genetic locus encoding *Haemophilus influenzae* type b surface fibrils. J. Bacteriol. 178:6281–6287.
62. **Stoker, N. G., N. F. Fairweather, and B. G. Spratt.** 1982. Versatile low-copy-number plasmid vectors for cloning in *Escherichia coli*. Gene 18:335–341.
63. **Tamm, A., A. Tarkkanen, T. K. Korhonen, P. Kuusela, P. Toivanen, and M. Skurnik.** 1993. Hydrophobic domains affect the collagen-binding specificity and surface polymerization as well as the virulence potential of the YadA protein of *Yersinia enterocolitica*. Mol. Microbiol. 10:995–1011.
64. **Tertti, R., M. Skurnik, T. Vartio, and P. Kuusela.** 1992. Adhesion protein YadA of *Yersinia* species mediates binding of bacteria to fibronectin. Infect. Immun. 60:3021–3024.
65. **Unhanand, M., I. Maciver, O. Ramilo, O. Arencibia-Mireles, J. C. Argyle, G. H. McCracken, Jr., and E. H. Hansen.** 1992. Pulmonary clearance of *Moraxella catarrhalis* in an animal model. J. Infect. Dis. 165:644–650.
66. **Verduin, C. M., H. J. Bootsma, C. Hol, A. Fleer, M. Jansze, K. L. Klingman, T. F. Murphy, and H. van Dijk.** 1995. Complement resistance in *Moraxella* (*Branhamella*) *catarrhalis* is mediated by a high-molecular-weight outer membrane protein (HMW-OMP), abstr. B-137, p. 189. *In* Abstracts of the General Meeting of the American Society for Microbiology 1995. American Society for Microbiology, Washington, D.C.
67. **Wald, E. R., D. D. Rohn, D. M. Chiponis, M. M. Blattner, K. S. Reisinger, and F. P. Wucher.** 1983. Quantitative cultures of middle-ear fluid in acute otitis media. J. Pediatr. 102:259–261.
68. **Wright, P. W., R. J. Wallace, Jr., and J. R. Shepherd.** 1990. A descriptive study of 42 cases of *Branhamella catarrhalis* pneumonia. Am. J. Med. 88(Suppl. 5A):2S–8S.
69. **Zaleska, M., K. Lounatmaa, M. Nurminen, E. Wahlstrom, and P. H. Makela.** 1985. A novel virulence associated cell surface structure composed of 47-kd protein subunits in *Yersinia enterocolitica*. EMBO J. 4:1013–1018.
70. **Zhang, H., R. Scholl, J. Browse, and C. Somerville.** 1988. Double-stranded DNA sequencing as a choice for DNA sequencing. Nucleic Acids Res. 16:1220.

Editor: P. E. Orndorff

☐ 1: AAB96359. high molecular we...[gi:8572547] BLink, Domains, Links

```
LOCUS       AAB96359                 832 aa            linear   BCT 19-JUN-2000
DEFINITION  high molecular weight outer membrane protein [Moraxella
            catarrhalis].
ACCESSION   AAB96359
VERSION     AAB96359.2  GI:8572547
DBSOURCE    locus MCU57551 accession U57551.2
KEYWORDS    .
SOURCE      Moraxella catarrhalis
  ORGANISM  Moraxella catarrhalis
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Moraxellaceae; Moraxella.
REFERENCE   1  (residues 1 to 832)
  AUTHORS   Aebi,C., Maciver,I., Latimer,J.L., Cope,L.D., Stevens,M.K.,
            Thomas,S.E., McCracken,G.H. Jr. and Hansen,E.J.
  TITLE     A protective epitope of Moraxella catarrhalis is encoded by two
            different genes
  JOURNAL   Infect. Immun. 65 (11), 4367-4377 (1997)
  MEDLINE   98013056
   PUBMED   9353007
REFERENCE   2  (residues 1 to 832)
  AUTHORS   Hansen,E.J.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-MAY-1996) Microbiology, University of Texas
            Southwestern Medical Center, 6000 Harry Hines Blvd., Dallas, TX
            75235, USA
REFERENCE   3  (residues 1 to 832)
  AUTHORS   Hansen,E.J.
  TITLE     Direct Submission
  JOURNAL   Submitted (19-JUN-2000) Microbiology, University of Texas
            Southwestern Medical Center, 6000 Harry Hines Blvd., Dallas, TX
            75235, USA
  REMARK    Sequence update by submitter
COMMENT     On Jun 19, 2000 this sequence version replaced gi:2772586.
            Method: conceptual translation.
FEATURES             Location/Qualifiers
     source          1..832
                     /organism="Moraxella catarrhalis"
                     /strain="O35E"
                     /db_xref="taxon:480"
     Protein         1..832
                     /product="high molecular weight outer membrane protein"
                     /name="UspA1"
     CDS             1..832
                     /gene="uspA1"
                     /coded_by="U57551.2:321..2819"
                     /transl_table=11
ORIGIN
```

```
      1 mnkiykvkkn aaghlvacse fakghtkkav lgsllivgal gmattasaqa tnskgtgahi
     61 gvnnnneapg dysfigsggy nkaegrysai ggglfnkatn eystivgggy nkaegrysti
    121 gggsnneatn eystivggdd nkatgrysti gggdnntaeg eystvaggkn nqatgtgsfa
    181 agvenqanae navavgkkni iegensvaig sentvktehk nvfilgsgtt gvtsnsvllg
    241 netagkqatt vknaevggls ltgfageska engvvsvgse ggerqivnvg agqisdtstd
    301 avngsqlhal atvvddnqyd ivnnradiln nqddikdlqk evkgldnevg elsrdinslh
    361 dvtdnqqddi kelkrgvkel dnevgvlsrd inslhddvad nqddiaknka dikglnkevk
    421 eldkevgvls rdigslhddv atnqadiakn qadiktlenn veeellnlsg rlldqkadid
    481 nninniyela qqqdqhssdi ktlknnveeg lldlsgrlid qkadiaknqa diaqnqtdiq
    541 dlaaynelqd qyaqkqteai dalnkassen tqniaknqad ianninniye laqqqdqhss
    601 diktlakvsa antdriaknk aeadasfetl tknqntlieq gealveqnka inqelegfaa
    661 hadiqdkqil qnqadittnk taieqninrt vangfeiekn kagiatnkqe lilqndrlnr
    721 inetnnrqdq kidqlgyalk eqgqhfnnri saverqtagg ianaiaiatl pspsragehh
    781 vlfgsgyhng qaavslgaag lsdtgkstyk iglswsdagg lsggvggsyr wk
//
```


Jul 30 2003 12:44:50

☐ 1: AAB96391. ubiquitous surfac...[gi:2735093] BLink, Domains, Links

```
LOCUS       AAB96391                 576 aa            linear   BCT 19-JUN-2000
DEFINITION  ubiquitous surface protein A 2 [Moraxella catarrhalis].
ACCESSION   AAB96391
VERSION     AAB96391.1  GI:2735093
DBSOURCE    locus MCU86135 accession U86135.2
KEYWORDS    .
SOURCE      Moraxella catarrhalis
  ORGANISM  Moraxella catarrhalis
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Moraxellaceae; Moraxella.
REFERENCE   1  (residues 1 to 576)
  AUTHORS   Aebi,C., Maciver,I., Latimer,J.L., Cope,L.D., Stevens,M.K.,
            Thomas,S.E., McCracken,G.H. Jr. and Hansen,E.J.
  TITLE     A protective epitope of Moraxella catarrhalis is encoded by two
            different genes
  JOURNAL   Infect. Immun. 65 (11), 4367-4377 (1997)
  MEDLINE   98013056
   PUBMED   9353007
REFERENCE   2  (residues 1 to 576)
  AUTHORS   Hansen,E.J., Aebi,C. and Cope,L.D.
  TITLE     Direct Submission
  JOURNAL   Submitted (21-JAN-1997) Microbiology, UT Southwestern Medical
            Center, 6000 Harry Hines Blvd., Dallas, TX 75235-9048, USA
REFERENCE   3  (residues 1 to 576)
  AUTHORS   Hansen,E.J., Aebi,C. and Cope,L.D.
  TITLE     Direct Submission
  JOURNAL   Submitted (19-JUN-2000) Microbiology, UT Southwestern Medical
            Center, 6000 Harry Hines Blvd., Dallas, TX 75235-9048, USA
  REMARK    Sequence update by submitter
COMMENT     Method: conceptual translation.
FEATURES             Location/Qualifiers
     source          1..576
                     /organism="Moraxella catarrhalis"
                     /strain="O35E"
                     /db_xref="taxon:480"
     Protein         1..576
                     /product="ubiquitous surface protein A 2"
                     /name="UspA2; high molecular weight outer membrane
                     protein"
     CDS             1..576
                     /gene="uspA2"
                     /coded_by="U86135.2:614..2344"
                     /transl_table=11
ORIGIN
        1 mktmkllplk iavtsamivg lgatstvnaq vveqffpnif fnenhdeldd ayhnmilgdt
       61 aivsnsqdns tqlkfysnde dsvpdsllfs kllheqqlng fkagdtiipl dkdgkpvytk
      121 dtrtkdgkve tvysvttkia tqddveqsay srgiqgdidd lydinrevne ylkathdyne
```

```
      181 rqteaidaln kassantdri dtaeeridkn eydikalesn veegllelsg hlidqkadlt
      241 kdikalesnv eegllelsgh lidqkadltk dikalesnve eglldlsgrl ldqkadiakn
      301 qadiaqnqtd iqdlaaynel qdayakqqte aidalnkass entqniaknq adianninni
      361 yelaqqqdqh ssdiktlaka saantdriak nkadadasfe tltknqntli ekdkehdkli
      421 tanktaidan kasadtkfaa tadaitkngn aitknaksit dlgtkvdgfd grvtaldtkv
      481 naldtkvnaf dgritaldsk vengmaaqaa lsglfqpysv gkfnataalg gygsksavai
      541 gagyrvnpnl afkagaaint sgnkkgsyni gvnyef
//
```


Jul 30 2003 12:44:50

Figure 1A

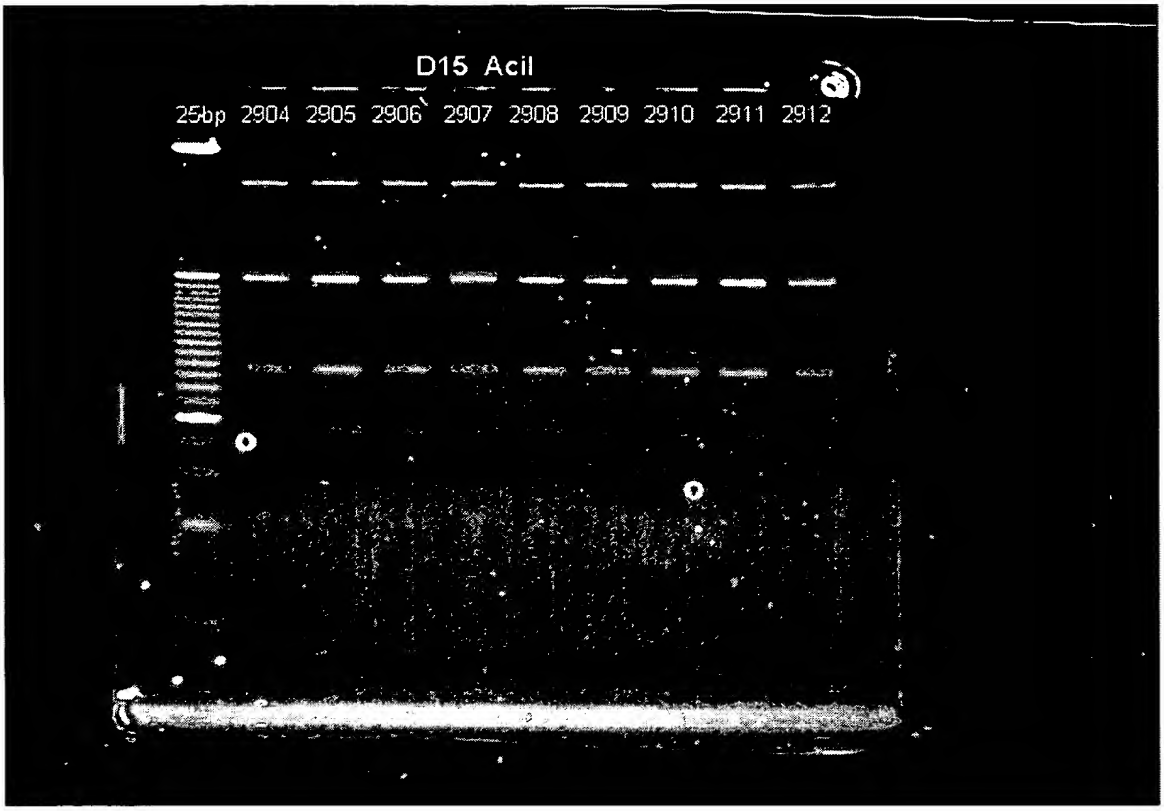


Figure 1B

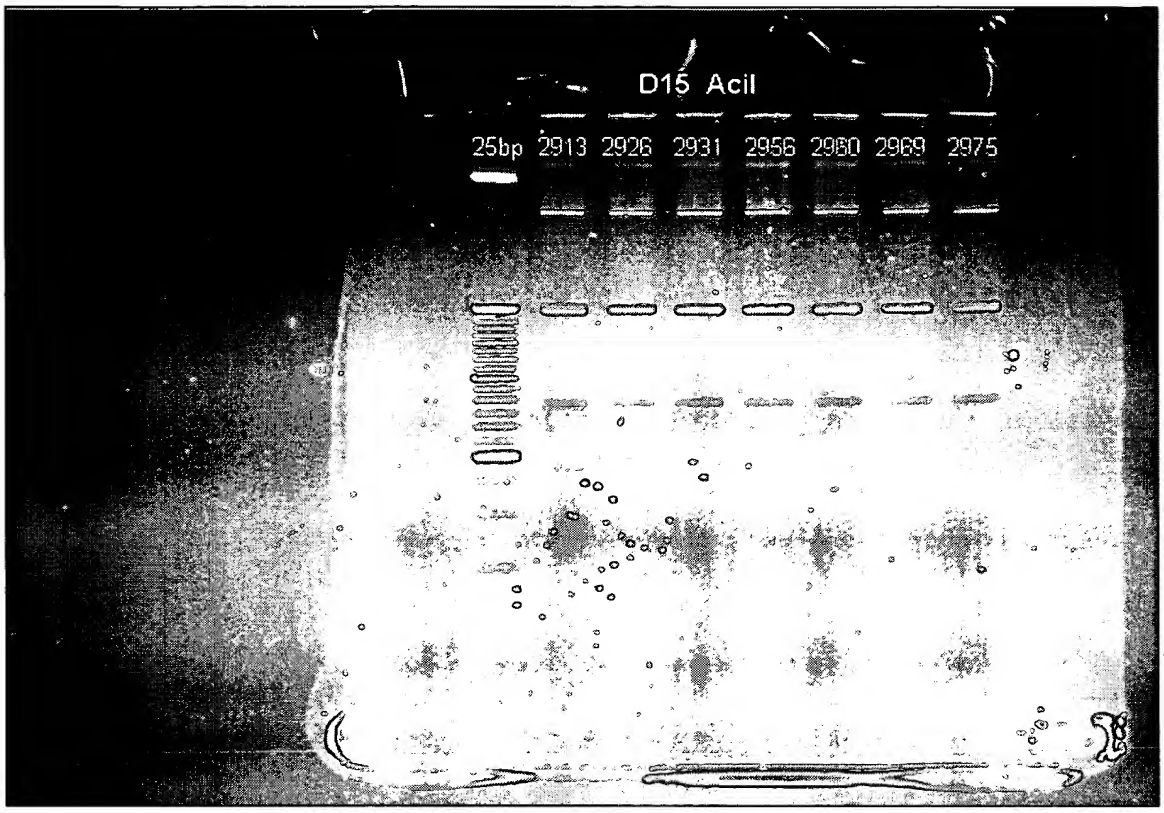


Figure 1C

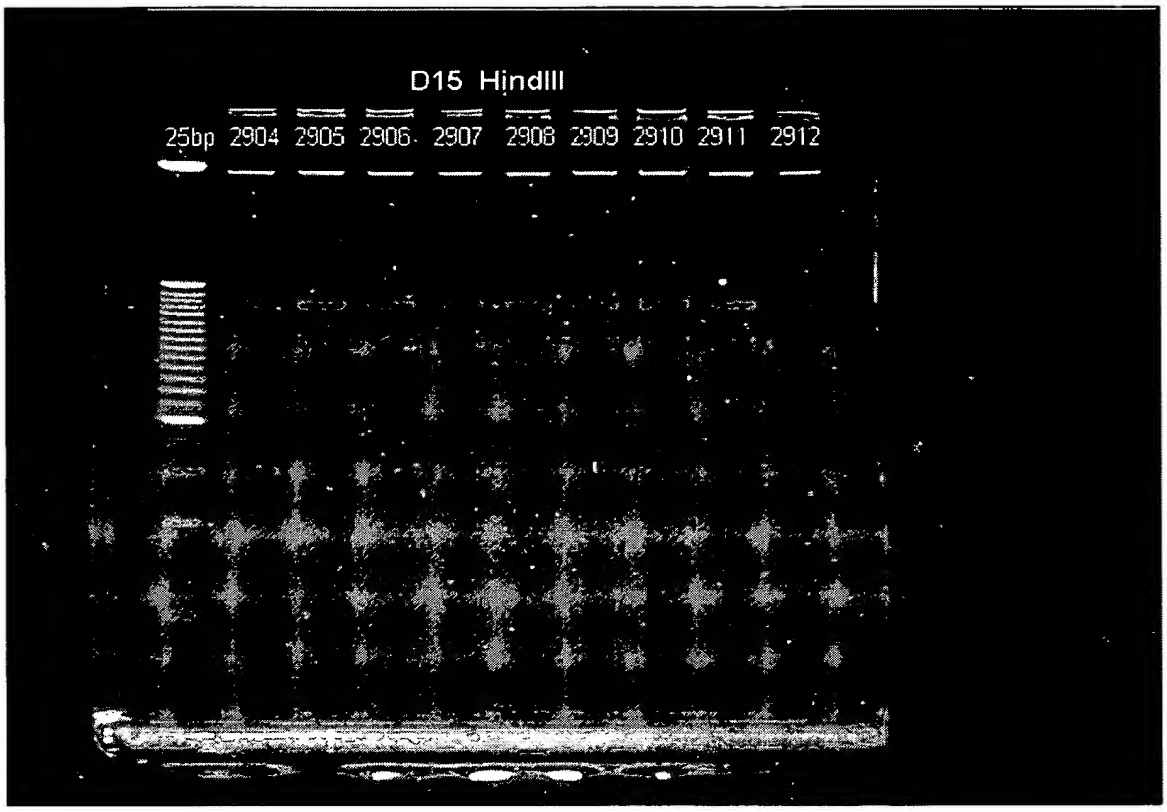


Figure 1D

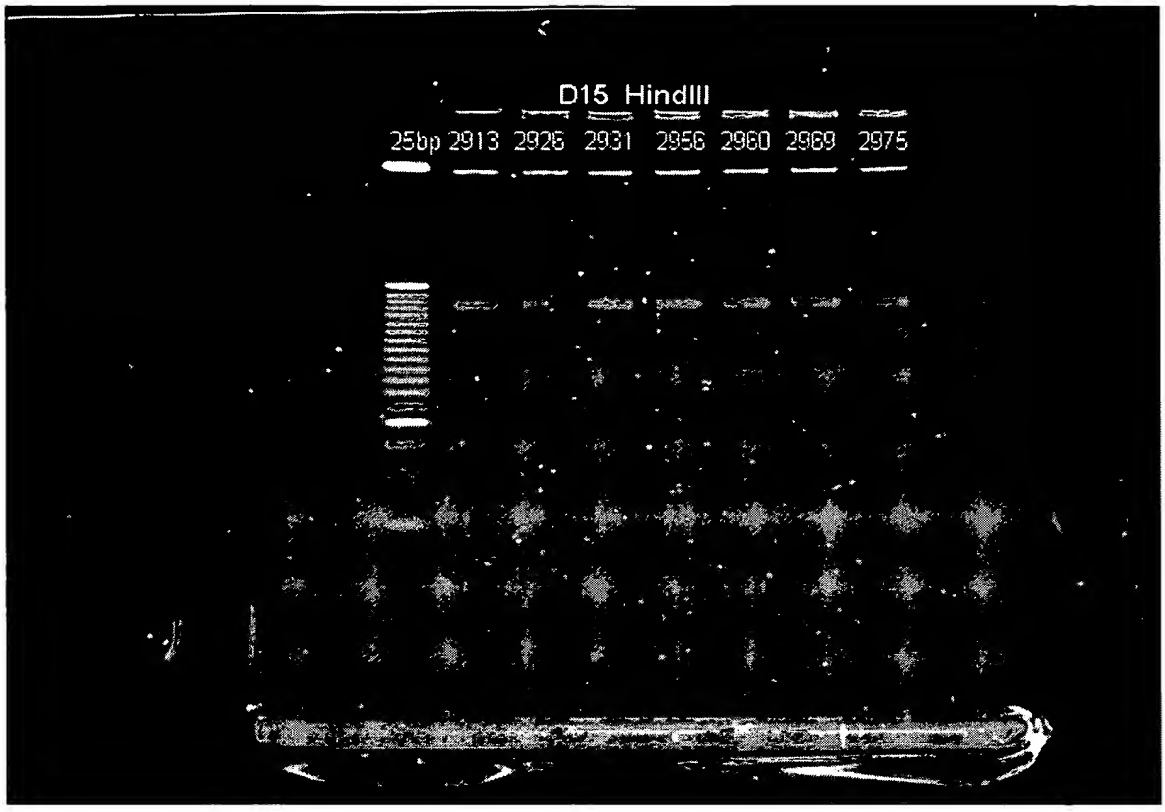


Figure 1E

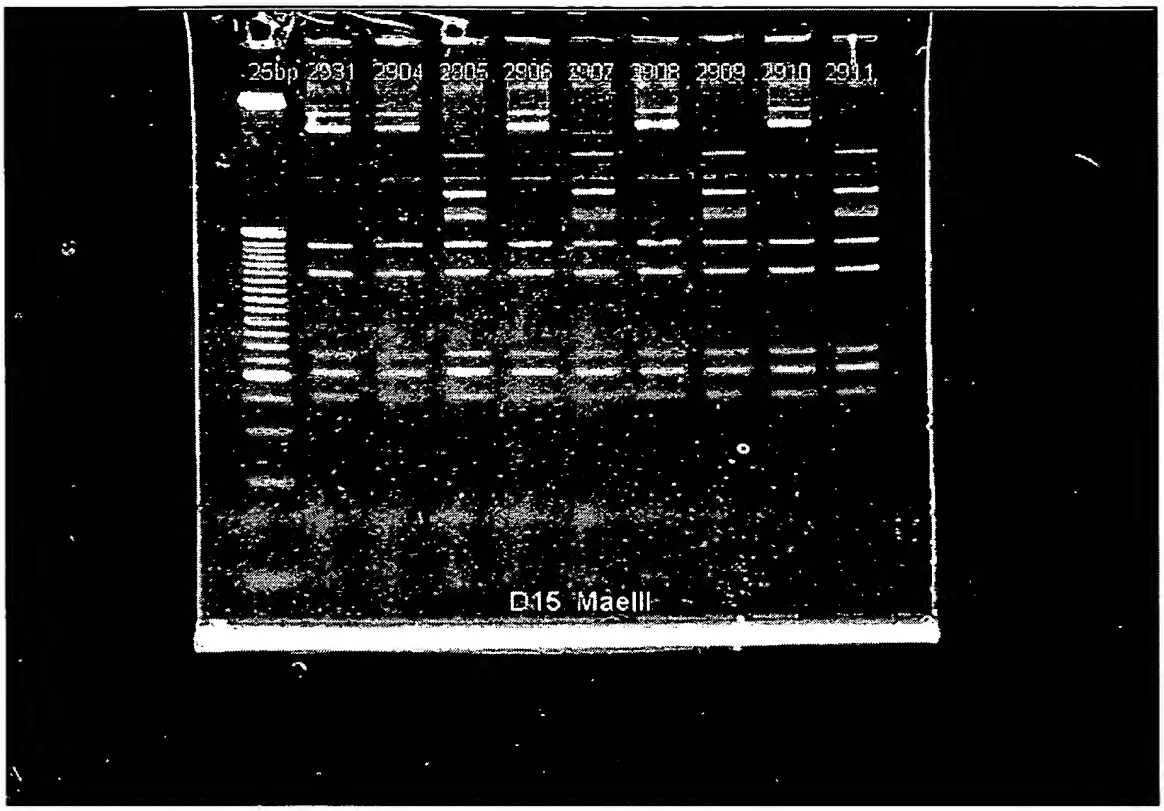


Figure 1F

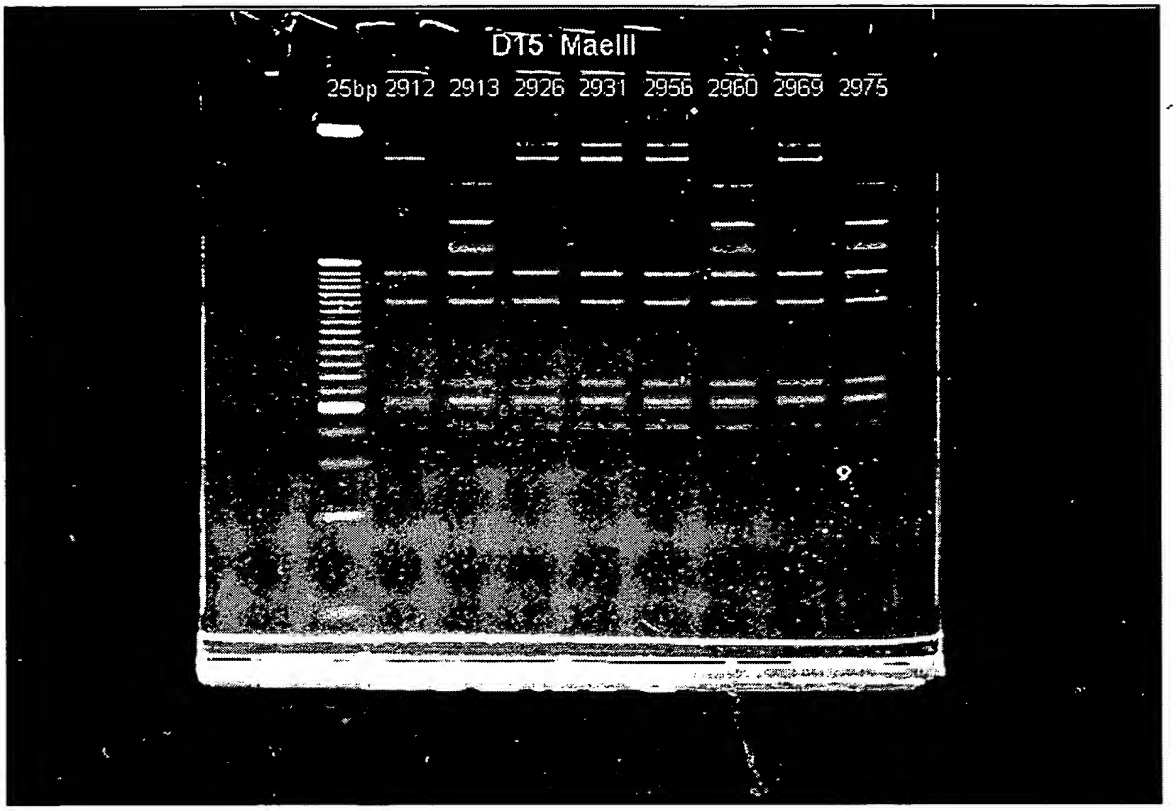


Figure 1G

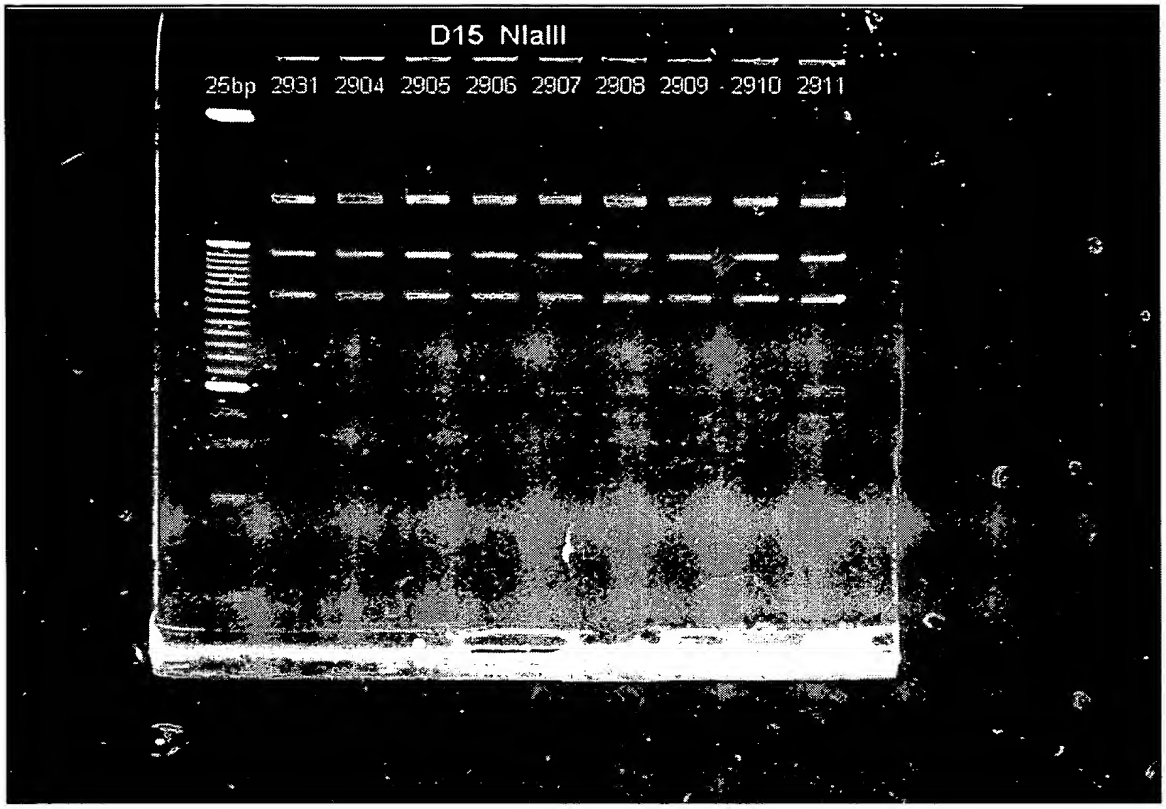


Figure 1H

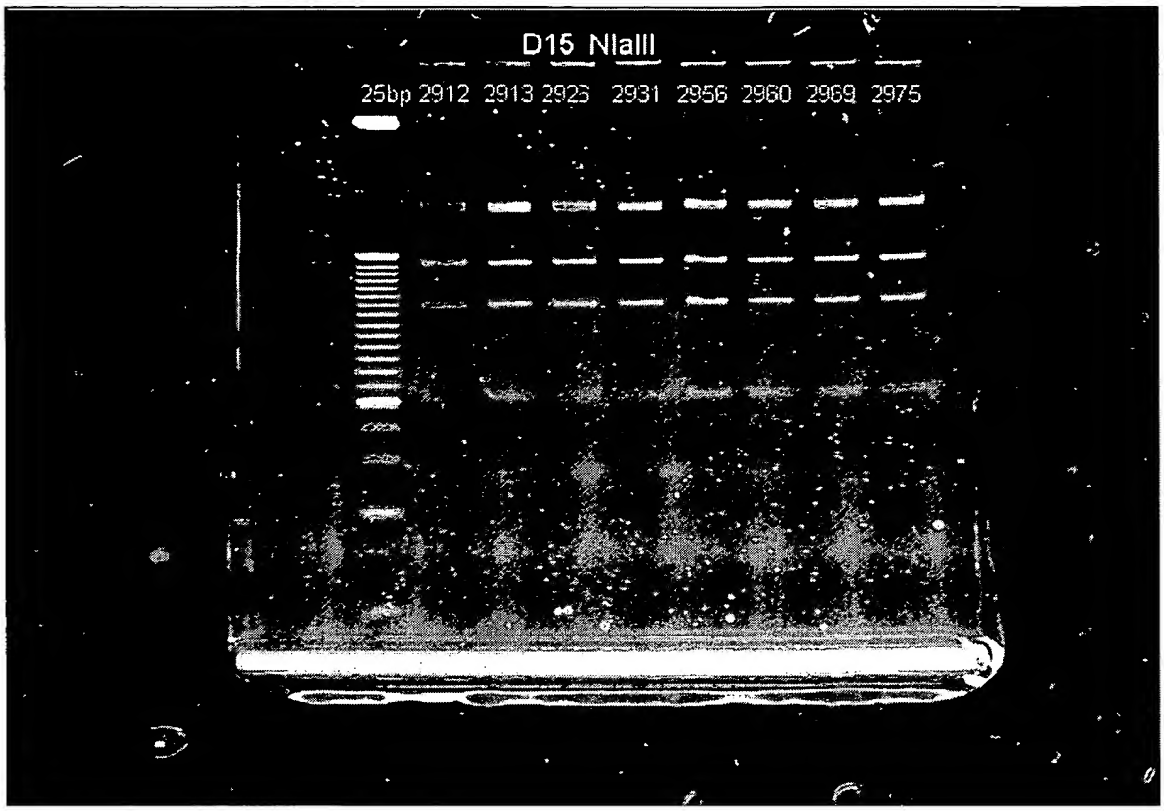


Figure 1I

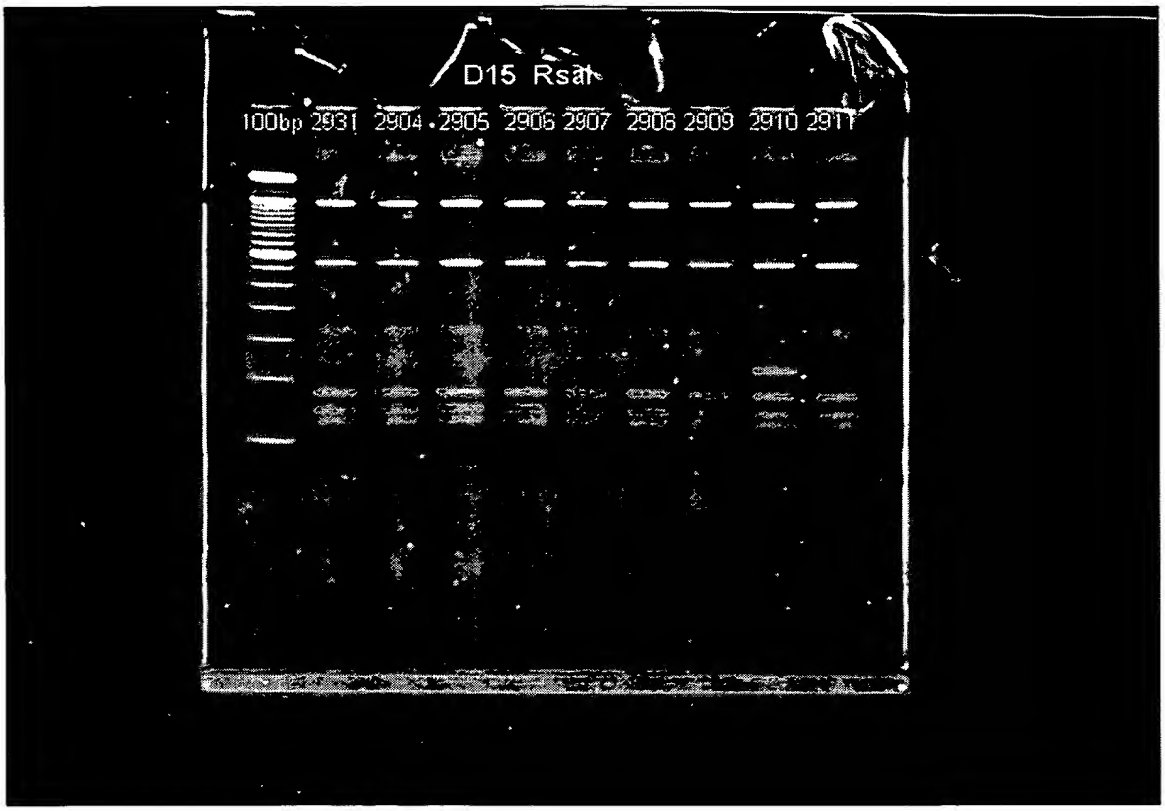


Figure 1J

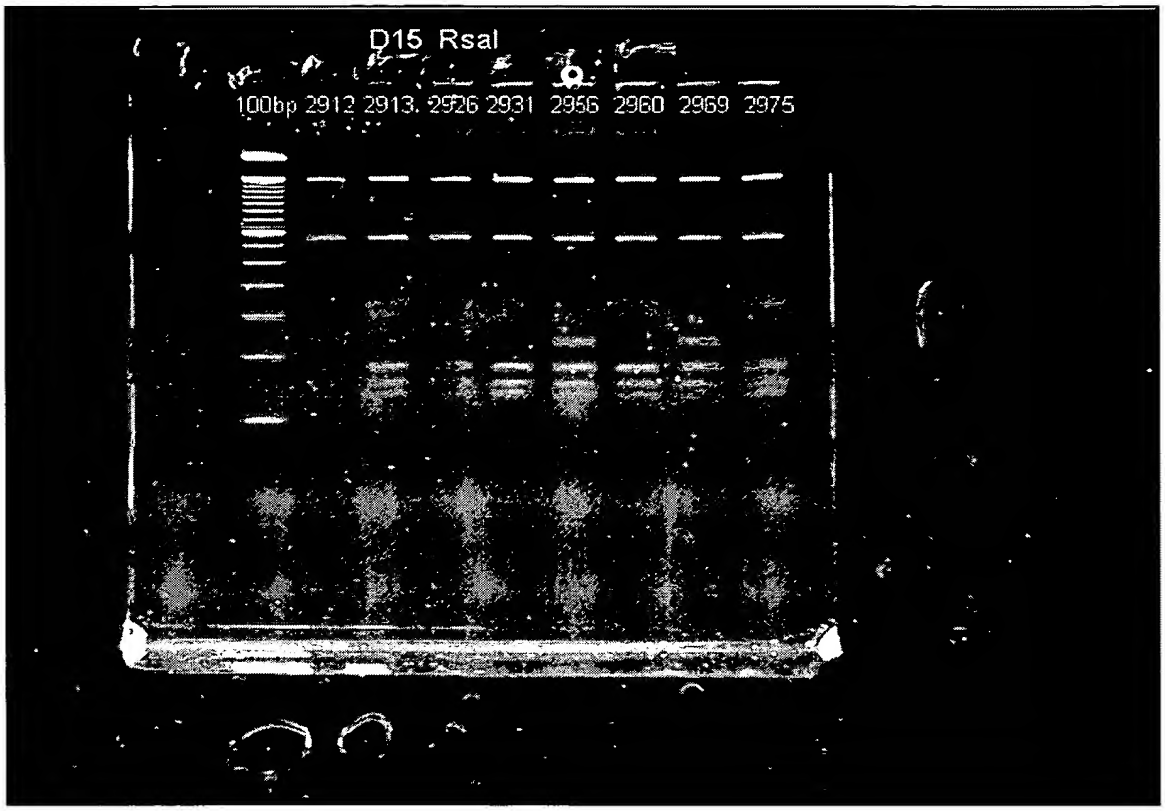


Figure 1K

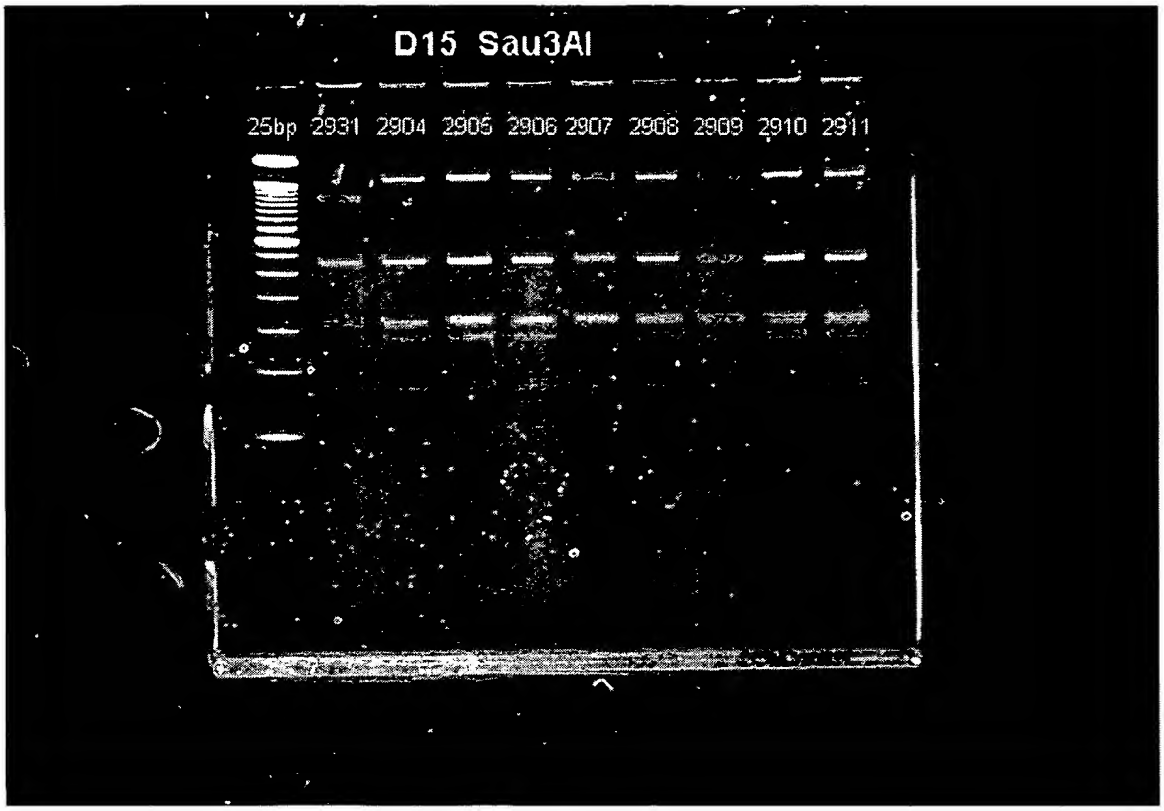


Figure 1L

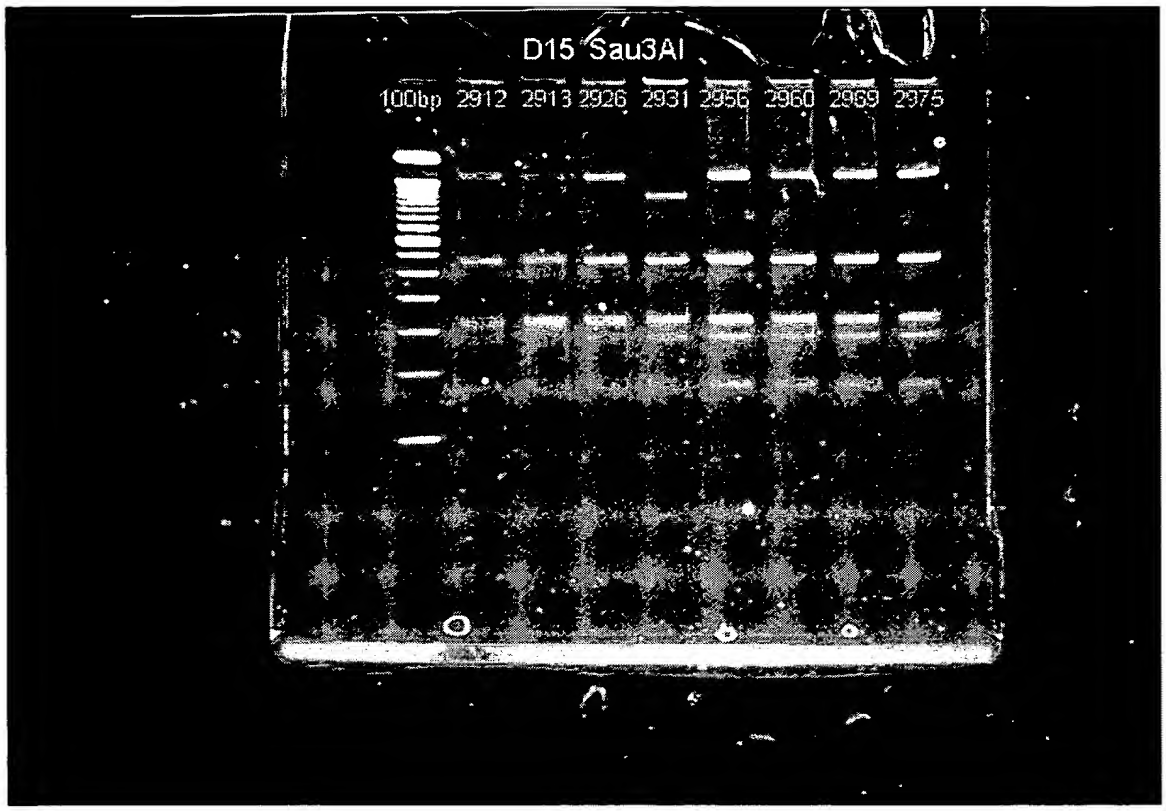


Figure 2A

Identity to SeqID No:1 is indicated by a dot.

```
                  *         20         *         40         *
Seqid1 : ATGCGTAATTCATATTTTAAAGGTTTTCAGGTCAGTGCAATGACAATGGC :   50
Seqid3 : ................................................... :   50

                60         *         80         *        100
Seqid1 : TGTCATGATGGTAATGTCAACTCATGCACAAGCGGCGGATTTTATGGCAA :  100
Seqid3 : ................................................... :  100

                  *        120         *        140         *
Seqid1 : ATGACATTACCATCACAGGACTACAGCGAGTGACCATTGAAAGCTTACAA :  150
Seqid3 : ........G.......................................... :  150

              160         *        180         *        200
Seqid1 : AGCGTGCTGCCGTTTCGCTTGGGTCAAGTGGTGAGCGAAAACCAGTTGGC :  200
Seqid3 : ..................................GCA.............. :  200

                  *        220         *        240         *
Seqid1 : TGATGGTGTCAAAGCACTTTATGCAACAGGCAATTTTTCAGATGTGCAAG :  250
Seqid3 : ................................................... :  250

              260         *        280         *        300
Seqid1 : TCTATCATCAAGAAGGGCGTATCATCTATCAGGTAACCGAAAGGCCGTTA :  300
Seqid3 : ................................................... :  300

                  *        320         *        340         *
Seqid1 : ATCGCTGAGATTAATTTTGAGGGCAATCGCTTAATTCCAAAAGAAGGTCT :  350
Seqid3 : ................................................... :  350
```

Figure 2B

```
                360         *         380         *         400
Seqid1 : ACAAGAAGGGCTAAAAAATGCTGGCTTAGCTGTGGGTCAACCACTAAAAC :  400
Seqid3 : ..................................................... :  400

                    *       420         *       440         *
Seqid1 : AAGCCACAGTACAGATGATCGAAACCGAGCTTACCAATCAATATATATCA :  450
Seqid3 : ..................................................... :  450

                460         *         480         *         500
Seqid1 : CAAGGCTATTATAATACCGAAATTACTGTCAAACAGACGATGCTTGATGG :  500
Seqid3 : ..................................................... :  500

                    *       520         *       540         *
Seqid1 : TAATCGTGTTAAGCTTGATATGACCTTTGCTGAAGGTAAACCTGCACGGG :  550
Seqid3 : ..................................................... :  550

                560         *         580         *         600
Seqid1 : TGGTTGATATTAATATCATTGGCAATCAGCATTTTAGCGATGCAGATTTG :  600
Seqid3 : ..................................................... :  600

                    *       620         *       640         *
Seqid1 : ATTGATGTGCTTGCGATTAAGGATAATAAAATCAATCCACTGTCTAAAGC :  650
Seqid3 : ..................................................... :  650

                660         *         680         *         700
Seqid1 : TGACCGTTATACTCAAGAAAAGCTGGTGACCAGTTTAGAGAATTTGCGTG :  700
Seqid3 : ..................................................... :  700

                    *       720         *       740         *
Seqid1 : CTAAATATCTCAATGCAGGGTTTGTGCGTTTTGAGATTAAAGATGCTAAG :  750
```

Figure 2C

```
Seqid3 : ..................................................... :  750

                760         *       780         *       800
Seqid1 : CTTAATATTAATGAAGATAAAAACCGTATCTTTGTTGAGATTTCATTGCA :  800
Seqid3 : ..................................................... :  800

                    *       820         *       840         *
Seqid1 : TGAAGGTGAGCAATATCGCTTTGGACAGACACAGTTTTTGGGTAATTTAA :  850
Seqid3 : ..................................................... :  850

                860         *       880         *       900
Seqid1 : CTTATACTCAAGCAGAACTTGAGGCACTGCTTAAATTCAAAGCAGAAGAA :  900
Seqid3 : ..................................................... :  900

                    *       920         *       940         *
Seqid1 : GGGTTTTCACAAGCCATGCTTGAGCAAACAACAAACAATATCAGTACCAA :  950
Seqid3 : ..................................................... :  950

                960         *       980         *      1000
Seqid1 : ATTTGGTGACGATGGCTATTATTATGCTCAAATCCGTCCTGTAACACGCA : 1000
Seqid3 : ..................................................... : 1000

                    *      1020         *      1040         *
Seqid1 : TTAATGATGAAAGTCGTACGGTTGATGTGGAATATTATATTGACCCTGTA : 1050
Seqid3 : ..................................................... : 1050

               1060         *      1080         *      1100
Seqid1 : CACCCTGTCTATGTACGCCGTATTAATTTTACAGGTAACTTTAAGACCCA : 1100
Seqid3 : ..................................................... : 1100
```

Figure 2D

```
                  *      1120         *      1140         *
Seqid1 : AGATGAAGTACTCCGTCGTGAGATGCGACAACTTGAAGGTGCGTTGGCAT : 1150
Seqid3 : ..................................................... : 1150

              1160         *      1180         *      1200
Seqid1 : CTAATCAAAAAATCCAGCTGTCTCGTGCACGCTTGATGCGGACTGGGTTT : 1200
Seqid3 : ..................................................... : 1200


                  *      1220         *      1240         *
Seqid1 : TTTAAACATGTTACCGTTGATACTCGTCCAGTACCCAACTCACCTGATCA : 1250
Seqid3 : ..................................................... : 1250

              1260         *      1280         *      1300
Seqid1 : GGTTGATGTAAATTTTGTGGTTGAAGAACAACCTTCAGGATCATCAACCA : 1300
Seqid3 : ..................................................... : 1300

                  *      1320         *      1340         *
Seqid1 : TCGCAGCAGGCTACTCTCAAAGTGGTGGTGTAACTTTTCAATTTGATGTT : 1350
Seqid3 : ..................................................... : 1350

              1360         *      1380         *      1400
Seqid1 : TCTCAAAATAACTTTATGGGTACAGGTAAGCACGTCAATGCTTCGTTTTC : 1400
Seqid3 : ..................................................... : 1400


                  *      1420         *      1440         *
Seqid1 : TCGCTCTGAGACCCGTGAGGTGTATAGTTTGGGTATGACCAACCCATACT : 1450
Seqid3 : ..................................................... : 1450


              1460         *      1480         *      1500
Seqid1 : TTACCGTAAATGGCGTCTCGCAAAGCTTGAGTGGCTACTATCGTAAAACC : 1500
Seqid3 : ..................................................... : 1500
```

Figure 2E

```
                  *      1520         *      1540         *
Seqid1 : AAGTATGATAACAAGAACATTAGTAATTATGTACTTGATTCTTATGGTGG : 1550
Seqid3 : ..................................................... : 1550

             1560         *      1580         *      1600
Seqid1 : CTCATTAAGCTATGGATATCCAATTGATGAAAATCAACGCATAAGCTTTG : 1600
Seqid3 : ..................................................... : 1600

                  *      1620         *      1640         *
Seqid1 : GTCTGAATGCTGACAATACCAAGCTTCATGGCGGTCGTTTTATGGGCATT : 1650
Seqid3 : ..................................................... : 1650

             1660         *      1680         *      1700
Seqid1 : AGTAATGTCAAGCAGCTGATGGCAGATGGTGGCAAAATTCAAGTGGATAA : 1700
Seqid3 : ..................................................... : 1700

                  *      1720         *      1740         *
Seqid1 : TAATGGCATTCCTGATTTTAAGCATGATTACACAACCTACAATGCCATTT : 1750
Seqid3 : ..................................................... : 1750

             1760         *      1780         *      1800
Seqid1 : TGGGGTGGAATTATTCAAGTCTAGATCGCCCTGTATTTCCAACCCAAGGC : 1800
Seqid3 : ..................................................... : 1800

                  *      1820         *      1840         *
Seqid1 : ATGAGTCATTCTGTAGATTTGACGGTTGGTTTTGGTGATAAAACTCATCA : 1850
Seqid3 : ..................................................... : 1850

             1860         *      1880         *      1900
Seqid1 : AAAAGTGGTTTATCAAGGCAATATCTATCGCCCATTTATCAAAAAATCAG : 1900
```

Figure 2F

```
Seqid3 : ..................................................... : 1900

                 *      1920          *      1940          *
Seqid1 : TCTTGCGTGGATACGCCAAGTTAGGCTATGGCAATAATTTACCATTTTAT : 1950
Seqid3 : ..................................................... : 1950


               1960          *      1980          *      2000
Seqid1 : GAAAATTTCTATGCAGGCGGCTATGGTTCGGTTCGTGGCTATGATCAATC : 2000
Seqid3 : ..................................................... : 2000


                 *      2020          *      2040          *
Seqid1 : CTCTTTGGGTCCACGCTCACAAGCCTATTTGACAGCTCGTCGTGGTCAAC : 2050
Seqid3 : ..................................................... : 2050


               2060          *      2080          *      2100
Seqid1 : AAACCACACTAGGAGAGGTTGTTGGTGGTAATGCTTTGGCAACTTTCGGC : 2100
Seqid3 : ..................................................... : 2100


                 *      2120          *      2140          *
Seqid1 : AGTGAGCTGATTTTACCTTTGCCATTTAAAGGTGATTGGATAGATCAGGT : 2150
Seqid3 : ..................................................... : 2150


               2160          *      2180          *      2200
Seqid1 : GCGTCCAGTGATATTCATTGAGGGCGGTCAGGTTTTTGATACAACAGGTA : 2200
Seqid3 : ..................................................... : 2200


                 *      2220          *      2240          *
Seqid1 : TGGATAAACAAACCATTGATTTAACCCAATTTAAAGACCCACAAGCAACA : 2250
Seqid3 : ..................................................... : 2250
```

Figure 2G

```
              2260         *       2280         *       2300
Seqid1 : GCTGAACAAAATGCAAAAGCAGCCAATCGCCCGCTACTAACCCAAGATAA : 2300
Seqid3 : ..................................................... : 2300

                    *       2320         *       2340         *
Seqid1 : ACAGTTGCGTTATAGTGCTGGTGTTGGTGCAACTTGGTATACGCCCATTG : 2350
Seqid3 : ..................................................... : 2350

              2360         *       2380         *       2400
Seqid1 : GTCCTTTATCTATTAGCTATGCCAAGCCATTGAATAAAAAACAAAATGAT : 2400
Seqid3 : ..................................................... : 2400

                    *       2420         *       2440
Seqid1 : CAGACCGATACGGTACAGTTCCAGATTGGTAGTGTCTTTTAA : 2442
Seqid3 : ..................................................... : 2442
```

Figure 3A

Identity to SeqID No:2 is indicated by a dot.

```
                 *        20         *        40         *
Seqid2 : MRNSYFKGFQVSAMTMAVMMVMSTHAQAADFMANDITITGLQRVTIESLQ :  50
Seqid4 : .....................................A..... :  50

               60         *        80         *       100
Seqid2 : SVLPFRLGQVVSENQLADGVKALYATGNFSDVQVYHQEGRIIYQVTERPL : 100
Seqid4 : ............A..................................... : 100

                *       120         *       140         *
Seqid2 : IAEINFEGNRLIPKEGLQEGLKNAGLAVGQPLKQATVQMIETELTNQYIS : 150
Seqid4 : ..................................................... : 150

              160         *       180         *       200
Seqid2 : QGYYNTEITVKQTMLDGNRVKLDMTFAEGKPARVVDINIIGNQHFSDADL : 200
Seqid4 : ..................................................... : 200

                *       220         *       240         *
Seqid2 : IDVLAIKDNKINPLSKADRYTQEKLVTSLENLRAKYLNAGFVRFEIKDAK : 250
Seqid4 : ..................................................... : 250

              260         *       280         *       300
Seqid2 : LNINEDKNRIFVEISLHEGEQYRFGQTQFLGNLTYTQAELEALLKFKAEE : 300
Seqid4 : ..................................................... : 300

                *       320         *       340         *
Seqid2 : GFSQAMLEQTTNNISTKFGDDGYYYAQIRPVTRINDESRTVDVEYYIDPV : 350
Seqid4 : ..................................................... : 350
```

Figure 3B

```
                360         *         380         *         400
Seqid2 : HPVYVRRINFTGNFKTQDEVLRREMRQLEGALASNQKIQLSRARLMRTGF : 400
Seqid4 : ..................................................... : 400

                *         420         *         440         *
Seqid2 : FKHVTVDTRPVPNSPDQVDVNFVVEEQPSGSSTIAAGYSQSGGVTFQFDV : 450
Seqid4 : ..................................................... : 450

                460         *         480         *         500
Seqid2 : SQNNFMGTGKHVNASFSRSETREVYSLGMTNPYFTVNGVSQSLSGYYRKT : 500
Seqid4 : ..................................................... : 500

                *         520         *         540         *
Seqid2 : KYDNKNISNYVLDSYGGSLSYGYPIDENQRISFGLNADNTKLHGGRFMGI : 550
Seqid4 : ..................................................... : 550

                560         *         580         *         600
Seqid2 : SNVKQLMADGGKIQVDNNGIPDFKHDYTTYNAILGWNYSSLDRPVFPTQG : 600
Seqid4 : ..................................................... : 600

                *         620         *         640         *
Seqid2 : MSHSVDLTVGFGDKTHQKVVYQGNIYRPFIKKSVLRGYAKLGYGNNLPFY : 650
Seqid4 : ..................................................... : 650

                660         *         680         *         700
Seqid2 : ENFYAGGYGSVRGYDQSSLGPRSQAYLTARRGQQTTLGEVVGGNALATFG : 700
Seqid4 : ..................................................... : 700

                *         720         *         740         *
Seqid2 : SELILPLPFKGDWIDQVRPVIFIEGGQVFDTTGMDKQTIDLTQFKDPQAT : 750
```

Figure 3C

```
Seqid4 : ................................................. : 750


                760         *        780         *        800
Seqid2 : AEQNAKAANRPLLTQDKQLRYSAGVGATWYTPIGPLSISYAKPLNKKQND : 800
Seqid4 : ................................................. : 800


                    *
Seqid2 : QTDTVQFQIGSVF : 813
Seqid4 : ............. : 813
```

Figure 4

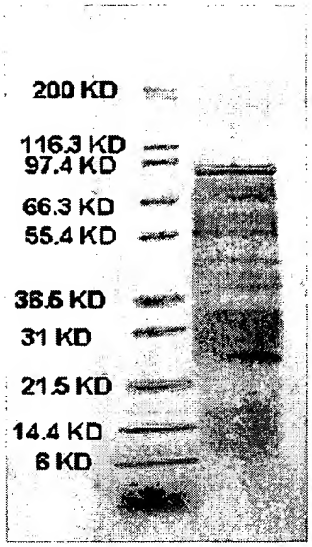


Figure 5

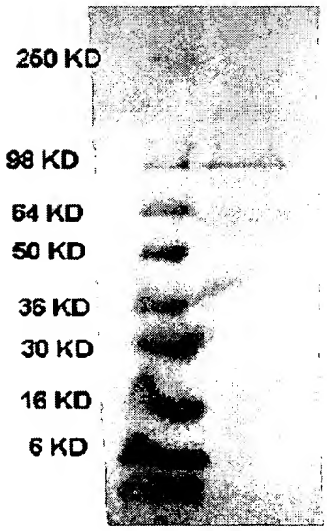


Figure 6

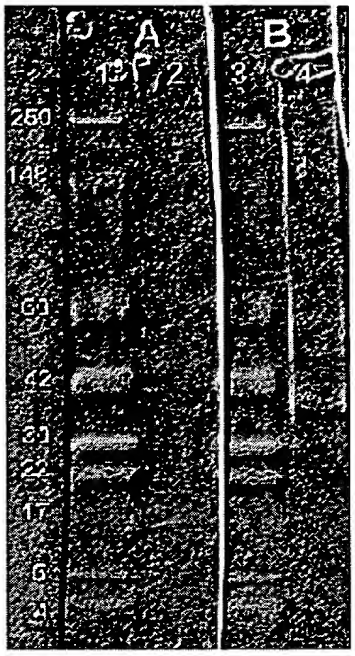


Figure 7A

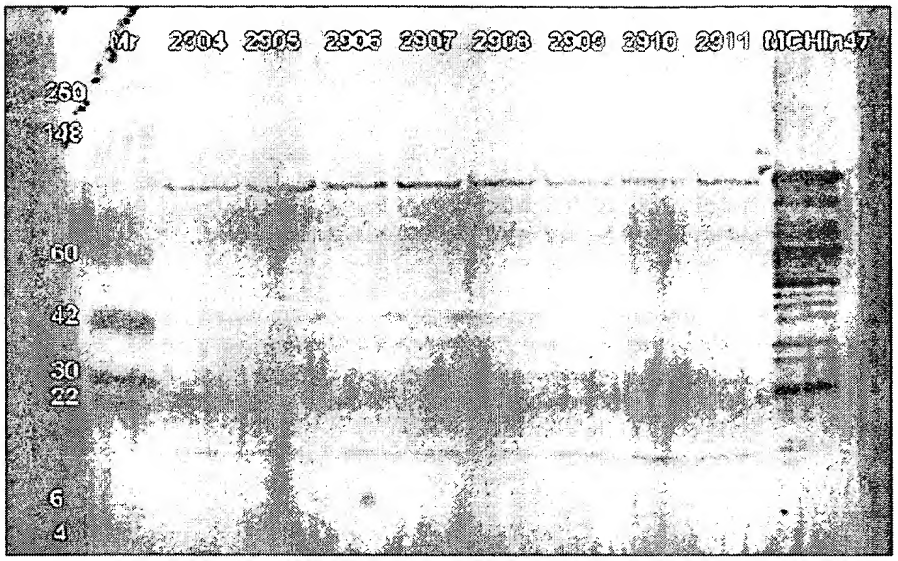


Figure 7B

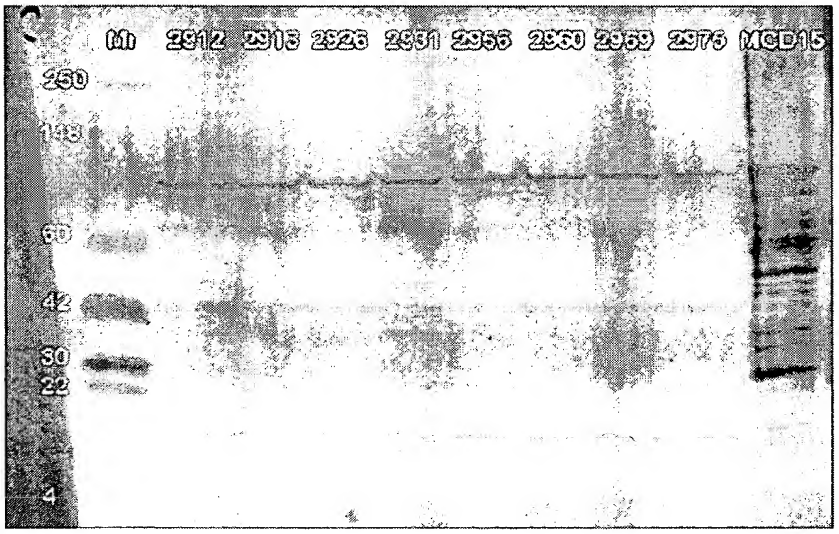
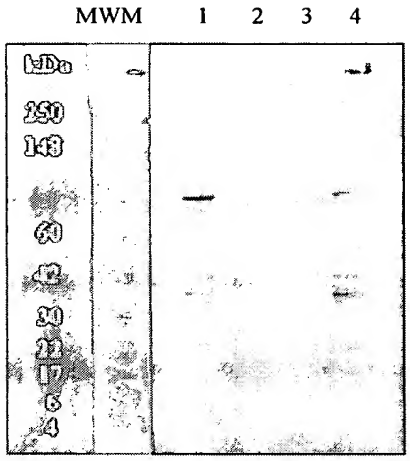


Figure 8



Possible Dissociation of the Heparin-binding and Mitogenic Activities of Heparin-binding (Acidic Fibroblast) Growth Factor-1 from Its Receptor-binding Activities by Site-directed Mutagenesis of a Single Lysine Residue

Wilson H. Burgess,* Anne M. Shaheen,* Mark Ravera,‡ Michael Jaye,‡ Patrick J. Donohue,* and Jeffrey A. Winkles*

*Laboratory of Molecular Biology, Jerome H. Holland Laboratory for the Biomedical Sciences, American Red Cross, Rockville, Maryland 20855; and ‡Rorer Biotechnology, Inc., King of Prussia, Pennsylvania 19406

Abstract. The fibroblast or heparin-binding growth factors (HBGFs) are thought to be modulators of cell growth and migration, angiogenesis, wound repair, neurite extension, and mesoderm induction. A better understanding of the structural basis for the different activities of these proteins should facilitate the development of agonists and antagonists of specific HBGF activities and identification of the signal transduction pathways involved in the mechanisms of action of these growth factors. Chemical modification studies of Harper and Lobb (Harper, J. W., and R. R. Lobb. 1988. *Biochemistry.* 27:671–678) implicated lysine 132 in HBGF-1 (acidic fibroblast growth factor) as being important to the heparin-binding, receptor-binding, and mitogenic activities of the protein. We changed lysine 132 to a glutamic acid residue by site-directed mutagenesis of the human cDNA and expressed the mutant protein in *Escherichia coli* to obtain sufficient quantities for functional studies. Replacement of this lysine with glutamic acid reduces the apparent affinity of HBGF-1 for immobilized heparin (elutes at 0.45 M NaCl vs. 1.1 M NaCl for wild-type). Mitogenic assays established two points: (*a*) human recombinant HBGF-1 is highly dependent on the presence of heparin for optimal mitogenic activity, and (*b*) the change of lysine 132 to glutamic acid drastically reduces the specific mitogenic activity of HBGF-1. The poor mitogenic activity of the mutant protein does not appear to be due to a reduced affinity for the HBGF receptor. Similarly, the mutant HBGF-1 can stimulate tyrosine kinase activity and induce protooncogene expression. Differences in the biological properties of the wild-type and mutant proteins were observed in transfection studies. Mutant HBGF-1 expression in transfected NIH 3T3 cells did not induce the same transformed phenotype characteristic of cells expressing wild-type HBGF-1. Together these data indicate that different functional properties of HBGF-1 may be dissociated at the structural level.

THE heparin-binding growth factor (HBGF)[1] family presently consists of seven structurally related polypeptides (3). The cDNAs for each have been cloned and sequenced. Two of the proteins, HBGF-1 and HBGF-2, have been characterized under many different names, but most often as acidic and basic fibroblast growth factor, respectively. Three sequence-related oncogenes have been identified: the hst oncogene was discovered based on its ability to transform NIH 3T3 cells (9, 25, 38, 45); the int-2 oncogene was first identified as a gene activated by mouse mammary tumor virus (7, 10, 11) and the FGF-5 oncogene was identified using NIH 3T3 transformation assays (46, 47). Recently a gene termed FGF-6 was identified by screening a mouse cosmid library with a human hst probe under reduced stringency and was shown to be capable of transforming NIH 3T3 cells (32). Finally, an epithelial cell-specific growth factor termed KGF or FGF-7 has been identified and its cDNA cloned and sequenced (13).

Functions associated with HBGF-1 and HBGF-2 include stimulation of mitogenesis, chemotaxis, mesoderm induction, neurite extension, and plasminogen activator activity. These HBGFs also induce angiogenesis in vivo and accelerate wound repair (for reviews see references 3, 18, 27, 36). The mechanisms by which HBGFs promote these functions are poorly understood but may include activation of protein tyrosine kinase activity (8, 15, 20), phosphorylation of phospholipase C-γ (6), and activation of immediate-early gene transcription (17). In addition, both HBGF-1 and HBGF-2 have been shown to be relatively resistant to degradation after internalization by receptor-mediated endocytosis (14, 24,

1. *Abbreviation used in this paper*: HBGF, heparin-binding growth factor.

34). Intact growth factor persists intracellularly for several hours and large fragments (15,000 and 10,000 M_r for HBGF-1; 16,000 M_r for HBGF-2) are detectable after as many as 24 h. Further, nuclear or nucleolar localization of HBGF-2 has been observed (2, 35).

Despite the identification of additional members of the HBGF family and a broad range of cells and tissues that contain the growth factors, and despite the availability of large quantities of recombinant protein and increased knowledge of the broad spectrum of activities of potential biological significance that can be attributed to the HBGFs, relatively little is known regarding the relationship of these highly conserved structures to any of their known functions. Baird et al. (1) reported the synthesis of 25 peptides, which together encompass and overlap the entire sequence of HBGF-2 as described by Ueno et al. (42). They reported the identification of two functional domains in the primary structure of HBGF-2 based on the abilities of synthetic peptides to interact with HBGF receptor, bind radiolabeled heparin in a solid phase assay, and inhibit HBGF-2 stimulation of thymidine incorporation into DNA. Using the numbering system of the authors (which does not correspond to full length HBGF-2) statistically significant functional activities could be assigned to peptides corresponding to residues 24–68 and 106–115 of HBGF-2. Similarly, Schubert et al. (39) demonstrated that peptides corresponding to residues 1–24, 24–68, and 93–120 of HBGF-2 are able to stimulate substratum adhesion of PC12 cells. We have shown that a synthetic peptide corresponding to residues 49–72 of HBGF-1 (using numbering of 1–154 for full length HBGF-1) is able to compete with HBGF-1 for heparin binding in a gel overlay assay (33). This region is homologous to one of the regions of HBGF-2 (residues 24–68) described above as possessing heparin-binding activity.

To date, the most complete and informative studies documenting the effects of chemical modification of any HBGF on function are those of Harper and Lobb (19). Briefly, they were able to show that limited reductive methylation of bovine HBGF-1 with formaldehyde and cyanoborohydride resulted in stoichiometric methylation only of lysine 132 (using 1–154 numbering for full length HBGF-1). They reported 90% modification of this residue, with 60% dimethylysine. The modified protein exhibited significantly reduced apparent affinity for immobilized heparin (eluted at ~0.7 M NaCl vs. ~1.2 M NaCl for unmodified HBGF-1), a fourfold reduction in its ability to stimulate DNA synthesis in NIH 3T3 fibroblasts and a similar reduction in its ability to compete with labeled ligand in a radioreceptor assay. A lysine residue is found at this position of HBGF-1 and HBGF-2 of all species characterized to date. Together these data implicate a crucial role for lysine 132 in several of the known functions of HBGF-1.

In this report we address the role of lysine 132 in HBGF-1 function using site-directed mutagenesis of this position to a glutamic acid. This approach offers several advantages over chemical modification studies including (*a*) the ability to produce large quantities of the desired product, (*b*) elimination of significant (although sub-stoichiometric) modification of other lysines, and (*c*) allowing the introduction of modified HBGF-1 into mammalian cells through transfection of cDNA expression vectors designed to produce the desired mutant. Despite these advantages the importance of chemical modification studies such as those of Harper and Lobb (19) should not be underestimated for they are extremely useful in the design of a rational approach to site-directed mutagenesis. The results described here demonstrate that replacement of lysine 132 of HBGF-1 with glutamic acid reduces significantly its apparent affinity for immobilized heparin and its mitogenic capacity. However, the apparent affinity of the mutant for high affinity cell surface receptors appears unaltered. When assayed in the presence of heparin where the difference in wild-type and mutant HBGF-1 mitogenic activity is most apparent, mutant HBGF-1 can stimulate tyrosine kinase activity and induce protooncogene expression. Functional differences between the wild-type and mutant HBGF-1 are also apparent after transfection of cDNA expression vectors into NIH 3T3 fibroblasts.

Materials and Methods

Materials

Heparin-Sepharose, protein A–Sepharose, pKK233 expression vectors, and low molecular weight markers were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). All reagents for PAGE and the Mighty Small Apparatus were from Hoefer Scientific Instruments (San Francisco, CA). Reagents for reversed-phase HPLC, amino acid analysis, and amino acid sequencing were purchased from Applied Biosystems, Inc. (Foster City, CA). Isotopes and the in vitro mutagenesis system were from Amersham Corp. (Arlington Heights, IL). The rabbit polyclonal HBGF-1–specific antibody was provided by R. Friesel (American Red Cross, Rockville, MD) and the rabbit polyclonal anti–phospholipase C-γ antibodies were provided by A. Zilberstein (Rorer Biotechnology, Inc., King of Prussia, PA). Tissue culture media and plasticware were purchased from Gibco Laboratories (Grand Island, NY). High molecular weight molecular markers were from Bio-Rad Laboratories (Richmond, CA). Endoproteinase ASP-N and the random primer DNA labeling kit were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Other chemicals were reagent grade.

Construction of pREC and p132E Prokaryotic Expression Plasmids

The plasmid expressing wild-type HBGF-1 (corresponding to the α-form of endothelial cell growth factor (5), pREC, was kindly provided by R. Forough (American Red Cross). This plasmid was constructed by cloning synthetic oligonucleotide cassettes into the Nco I/Hind III site of pKK233-2. The plasmid expressing mutant HBGF-1 (glutamic acid instead of lysine at amino acid position 132; p132E) was constructed as follows. The Eco RI/Hind III fragment of HBGF-1 cDNA clone 1 (21) was subcloned into M13mp18. Single-stranded template was prepared and used for oligonucleotide-directed in vitro mutagenesis. Double-stranded DNA was transformed into *E. coli* TG-1 cells and the resultant plaques were screened by M13 dideoxy sequencing. The mutated HBGF-1 cDNA was transferred into the expression vector pKK223-3 using the original Eco RI and Hind III sites.

Production and Purification of Recombinant Proteins

Recombinant plasmids pREC or p132E were introduced into the *lacI*^{q}-bearing *Escherichia coli* strain JM103. Cultures of JM103 bearing the recombinant plasmids were grown with shaking at 37°C in Luria broth containing 100 μg/ml ampicillin. A fresh overnight culture was diluted and grown until the A_{550} reached ~0.2, at which point isopropylthio-β-galactoside was added to 1 mM. Cells were collected by centrifugation and frozen at −80°C for subsequent growth factor purification.

The frozen cell pellets from 2-liter cultures were resuspended in 50 ml of 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 50 mM glucose. A fresh solution of hen egg lysozyme in the same buffer was added to 10 μg/ml. The cells were mixed at 4°C for 45 min. The viscous lysate was sonicated at maximum intensity using a large probe and four 20-s pulses of a Heat Systems W-380 sonicator. The lysate was clarified by centrifugation at 6,000 *g* for 15 min at 4°C. The supernatant was diluted to 100 ml with 50 mM Tris-HCl, pH 7.5, 10 mM EDTA and incubated with 20 ml of hydrated heparin-

Sepharose at 4°C with end-over-end mixing for 2 h. The resin was eluted batchwise using a sintered glass funnel and successive washes of the same buffer containing 0, 0.1, 0.5, 0.65, and 1.5 M NaCl.

The wild-type recombinant HBGF-1 eluted with the 1.5 M NaCl wash. The mutant was eluted with the 0.5 M NaCl wash. Although the wild-type protein was essentially pure after heparin-Sepharose chromatography, the mutant HBGF-1 constituted only 10–20% of the 0.5 M NaCl wash. Both preparations were purified to >95% purity using reversed-phase HPLC (4). The reversed-phase purified material was used for all reported studies.

Characterization of Recombinant Proteins

All preparations of purified recombinant human wild-type and mutant HBGF-1 were analyzed by SDS-PAGE, amino acid analysis, amino terminal sequencing, peptide mapping, and amino acid sequencing of the peptide encompassing the mutated residue. Protein concentrations were determined by amino acid analysis. Aliquots of wild-type and mutant HBGF-1 were subjected to electrophoresis using the SDS-PAGE system of Laemmli (26). A 15% acrylamide, 0.4% *N,N'*-methylenebisacrylamide solution was polymerized in a Hoefer mini-gel apparatus and electrophoresis was carried out at a constant 200 V. Protein was visualized by staining the gel with 0.1% Coomassie blue R-250 in 50% methanol, 10% glacial acetic acid, and destaining with 9% glacial acetic acid, 5% methanol. Samples for amino acid analysis were hydrolyzed with argon-purged, constant boiling 6 N HCl at 115°C for 18 h using a Pico-Tag workstation (Waters Associates, Milford, MA). Amino acids were derivatized with phenylisothiocyanate and separated with a PTC analyzer (model 130A; Applied Biosystems, Inc.). A Waters 840 system was used for data collection and reduction. Amino acid sequences were established using a protein sequencer (model 477A; Applied Biosystems, Inc.) using modified Edman chemistry and an on-line model 120A PTH analyzer. Peptide mapping of recombinant protein after digestion with endoproteinase Asp-N at a 1:25 ratio of enzyme to protein in 50 mM Na_2HPO_4, pH 8.0, 37°C for 18 h was performed using a microbore HPLC system (model 130A; Applied Biosystems, Inc.). The appropriate peptides were subjected to amino acid sequence analysis to establish the fidelity of expression of the wild-type and mutant HBGF-1 vectors.

Stability Studies

Metabolically labeled recombinant proteins were prepared by growing bacterial cultures as described above until the A_{550} reached ~0.4, at which point the cells were collected by centrifugation. They were resuspended in 98.5% M9 minimal medium/1.5% Luria broth and [^{3}H]leucine (140 Ci/mmol) was added to 45 μCi/ml. Cells were grown with shaking for 30 min, and then for an additional 4 h in the presence of 1 mM isopropylthio-β-galactoside. Cells were collected and growth factors purified as described above. The purified, labeled growth factors were incubated for 48 h at 37°C in the presence of media (DMEM containing 10% calf serum) that had been conditioned for 48 h by NIH 3T3 cells. The growth factor–containing media was analyzed by SDS-PAGE and autoradiography.

Mitogenic Assays

The mitogenic activities of wild-type and mutant recombinant HBGF-1 were determined by measuring their ability to stimulate DNA synthesis in NIH 3T3 cells and to support the proliferation of human umbilical vein endothelial cells. DNA synthesis was determined by measuring the amount of [^{3}H]thymidine incorporated into cells. Briefly, NIH 3T3 cells were seeded into 48-well plates and grown to near confluence in DME containing 10% calf serum. The cells were serum starved (DME, 0.5% calf serum) for 24 h. Mitogens were added to the wells and incubated for 18 h. The cells were pulsed with 0.5 μCi/ml of [^{3}H]thymidine (25 Ci/mmol) for 4 h. The cells were rinsed with PBS, fixed with 10% TCA, rinsed with PBS, and then solubilized with 0.5 N NaOH. Incorporation of [^{3}H]thymidine into acid-insoluble material was determined by scintillation counting. All assays were performed in triplicate.

Human umbilical vein endothelial cells were provided by T. Maciag (American Red Cross, Rockville, MD). They were maintained on fibronectin-coated plates (2 μg/cm^2) in medium 199 supplemented with 10% (vol/vol) heat-inactivated FBS, 1× antibiotic-antimycotic, 10 U/ml heparin, and 10 ng/ml human recombinant HBGF-1. For growth assays, cells were seeded in 24-well plates at 2,000 cells/well in medium 199 supplemented as above with the exception of HBGF-1. The indicated amounts of wild-type or mutant HBGF-1 and heparin were added to the wells. The media was changed ever other day. After 7 d in culture, cells were trypsinized and counted using a hemocytometer.

Competition for Binding and Cross-Linking to Cell Surface Receptors

Bovine brain–derived HBGF-1 (4) was labeled with ^{125}I using immobilized lactoperoxidase and biologically active, labeled protein was isolated using heparin-Sepharose as described (16). Confluent NIH 3T3 cells in 24-well plates were serum starved for 24 h before binding experiments in DME containing 0.5% calf serum. The cells were washed and incubated with DME containing 5 U/ml heparin, 0.5% BSA, and 25 mM Hepes, pH 7.2 (binding buffer) at room temperature for 20 min. The cells then were incubated with ^{125}I-HBGF-1 and unlabeled wild-type or mutant HBGF-1 in the presence of 5 U/ml heparin as indicated in the figure legend. The cells were incubated on ice for 90 min. The plates were aspirated and washed four times with binding buffer. The cells were then incubated for 20 min at 4°C with 1 ml of 0.3 mM disuccinimidyl suberate in PBS. The cross-linker was then aspirated off and the reaction quenched by adding 2.0 M Tris-HCl, pH 8.0. The cells were washed with PBS, scraped from the plates and pelleted for 10 s at 15,000 *g*. The pellets were extracted with 100 μl of 50 mM Tris, 1 mM EDTA, 200 mM NaCl, 1.0% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.5 for 20 min at 4°C. The extracts were centrifuged for 10 min at 15,000 *g*. The supernatants were removed and mixed with an equal volume of Laemmli sample buffer for SDS-PAGE analysis.

Stimulation of Protein Tyrosine Kinase Activity

NIH 3T3 cells were grown to confluence in 100 mm dishes and serum starved as described above. The cells were then exposed to diluent 1.0, or 10 ng/ml of wild-type or mutant HBGF-1 for 10 min at 37°C. The cells were washed once with cold PBS then lysed in buffer containing 10 mM Tris, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1.0% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, pH 7.4. The cells were scraped from the plates, vortexed, and incubated on ice for 10 min. Lysates were clarified by centrifugation at 10,000 *g* for 10 min at 4°C and the supernatants were mixed with an equal volume of 2× Laemmli sample buffer. Samples (normalized to cell number) were subjected to PAGE in the presence of SDS. The proteins were transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine antibodies as described (15). The blots were incubated with ^{125}I-protein A and phosphotyrosine-containing proteins were visualized by autoradiography. In some experiments the initial cell lysates were incubated with a pre-bound anti-phospholipase C-γ antibody/protein A–Sepharose complex (31) for 90 min at 4°C. The beads were washed with 20 mM Hepes, 0.1% Triton X-100, 150 mM NaCl, 10% glycerol, pH 7.5. Immunoprecipitated proteins were eluted from the beads with 2× Laemmli sample buffer and subjected to PAGE and Western blotting with anti-phosphotyrosine antibodies as described above.

RNA Gel Blot Analysis

NIH 3T3 cells were incubated for 48 h in DME/0.5% FCS and then either left unstimulated or stimulated with wild-type or mutant HBGF-1 for the indicated times. Cells were harvested, total RNA was prepared (17), and 10 μg of each sample was separated by electrophoresis on 1.2% agarose gels containing formaldehyde. The gels were stained with ethidium bromide photographed to verify that each lane contained an equal amount of undegraded ribosomal RNA. RNA was electroblotted onto Zetabind nylon filters and cross-linked by UV irradiation. The restriction fragments used and source of the DNA probes were as follows: (*a*) c-*fos*, 2.8-kb Nco I/Xho I fragment of pc-*fos*-1; American Type Culture Collection, Rockville, MD; (*b*) c-*jun*, 1.5-kb Hind III/Bam HI fragment of ph-cJ-1; gift of P. Angel, University of California, La Jolla, CA; (*c*) c-*myc*, 1.4-kb Sst I fragment of pHSR-1; ATCC; (*d*) glyceraldehyde 3-phosphate dehydrogenase, 0.8-kb Pst I/Xba I fragment of pHcGAP; ATCC. The probes were labeled with [^{32}P]dCTP (3,000 Ci/mmol) using a random primer labeling kit. Hybridization and filter washes were as described (17). Blots were exposed to Kodak XAR5 film at −70°C

Transfection of NIH 3T3 Cells with HBGF-1 Eukaryotic Expression Plasmids

NIH 3T3 cells in 100 mm dishes were transfected with plasmid DNA by the calcium phosphate precipitation method (44). Cells were incubated with either 1 μg of pSV2 neo (41) or co-transfected with a mixture (1:10 μg) of pSV2 neo and either HBGF-1 wild-type expression vector (p267) or HBGF-1 mutant expression vector (p268). The plasmid p267 is described in Jaye et

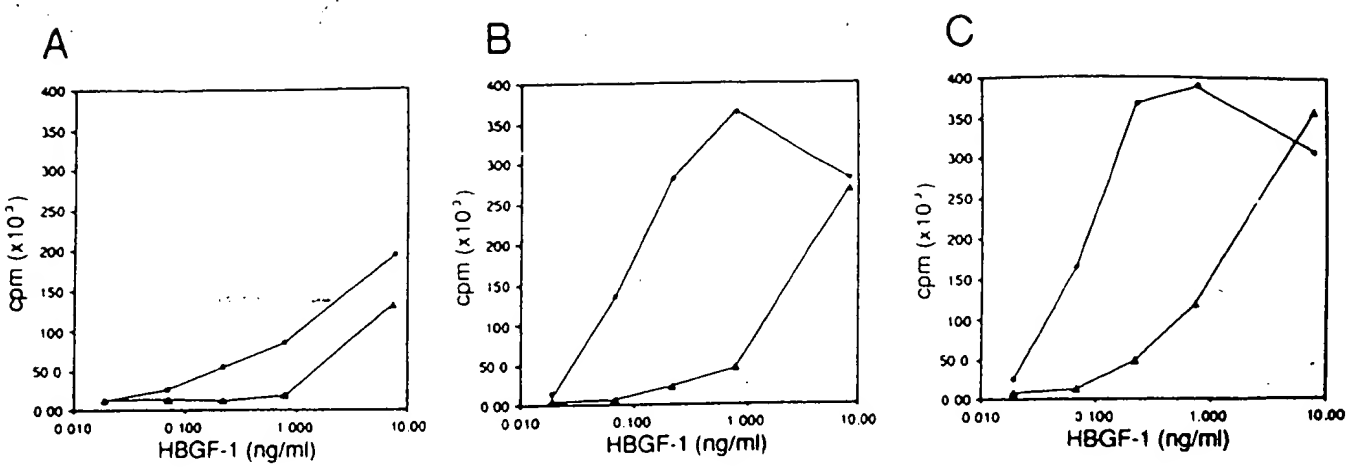


Figure 1. Stimulation of DNA synthesis in NIH 3T3 cells by wild-type and mutant HBGF-1. Cells were grown to near confluence and serum starved for 24 h as described in Materials and Methods. Cells were treated with the indicated concentrations of wild-type (●) or mutant (▲) HBGF-1, incubated for 18 h, and then pulsed with 0.5 μCi of [³H]thymidine/ml for 4 h. The cells were harvested and incorporation of radioactivity was determined. Both wild-type and mutant HBGF-1 were assayed in the presence of 0 (*A*), 5 (*B*), or 50 U/ml heparin (*C*).

al. (23); p268 was constructed by replacing the 297nt Pvu II/Bgl II fragment of p267 (encoding amino acids 38–155) with the corresponding region from the prokaryotic expression plasmid pEI32 using standard subcloning methods. Cells were split to 10 dishes and transfected colonies were selected by incubating the cells in DME, 10% calf serum containing 500 μg/ml Geneticin. The media was changed every 3–4 d. After 4 wk, transfected colonies were analyzed for HBGF-1 expression by Western blot analysis using rabbit polyclonal HBGF-1-specific antibodies and ^{125}I-protein A as described above.

Results

Heparin-binding Properties of HBGF-1 Mutant p132E

A drastic reduction in the apparent affinity of HBGF-1 containing glutamic acid in place of lysine at position 132 was observed during the purification of the recombinant proteins from the *Escherichia coli* lysates. Recombinant wild-type HBGF-1 from *E. coli* lysates can be purified to near homogeneity with a single heparin-Sepharose step. The protein binds the immobilized heparin during extensive washing with 0.5 and 0.65 M NaCl–containing buffers and is eluted with a single step of 1.5 M NaCl–containing buffer. In contrast, heparin-Sepharose affinity-based chromatography could not be used as a single purification step for the mutant HBGF-1. The mutant protein binds immobilized heparin in the presence of 0.1 M NaCl but was eluted during the 0.5 M NaCl wash. Both wild-type and mutant HBGF-1 (1.5 and 0.5 M NaCl eluates, respectively) could be purified to apparent homogeneity using reversed-phase HPLC. Detailed analysis of the apparent affinities of the two purified proteins for immobilized heparin-Sepharose using relatively shallow, linear NaCl gradients indicated that the mutant HBGF-1 eluted with 0.45 M NaCl whereas wild-type required 1.1 M NaCl to be eluted (data not shown). For all of the assays described below we used reversed-phase HPLC purified wild-type or mutant HBGF-1. Protein concentrations were determined by amino acid analysis of preparations that had been shown to be the desired HBGF-1 form by peptide mapping and amino acid sequence analysis (data not shown).

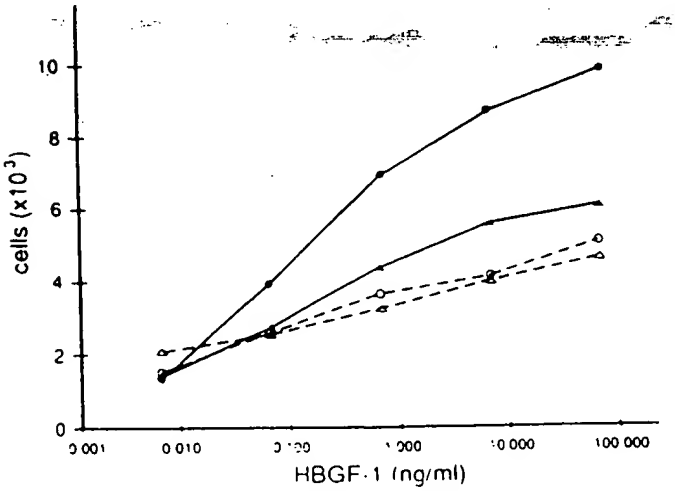


Figure 2. Ability of wild-type and mutant HBGF-1 to stimulate growth of human umbilical vein endothelial cells. Cells were seeded and cultured as described in Materials and Methods. Cell number after 7 d in culture in the presence of the indicated concentrations of wild-type (○/●) or mutant (△/▲) HBGF-1 in the absence (○/△) or presence (●/▲) of 50 U/ml heparin is shown.

Mitogenic Properties of HBGF-1 Mutant p132E

The ability of the HBGF-1 mutant to stimulate mitogenesis was compared to that of the wild-type protein using two different assays. In the first, the ability of the two proteins to stimulate DNA synthesis in NIH 3T3 cells as measured by [³H]thymidine incorporation was examined. The assays were conducted over a broad range of HBGF-1 and heparin concentrations. Two important points can be made from the data in Fig. 1. One, the wild-type HBGF-1 has a dramatic requirement for the presence of heparin for optimal mitogenic activity and, two, the mutant HBGF-1 is significantly less potent than wild-type protein in the presence of added heparin. As can be seen in Fig. 1, the maximal difference in mitogenic potency was observed in the presence of 5 U/ml heparin (~30-fold). Little difference (approximately three-

Table I. Cell Number ($\times 10^{-5}$)

	Growth factor concentration (ng/ml)					
	0	0.1	0.5	1	5	10
$GLU_{[1]}$ HBGF-1	1.6	1.6	1.3	1.2	1.7	1.4
Wild-type HBGF-1	1.7	2.0	1.9	2.9	12.6	16.6

fold) between the wild-type and mutant protein is seen in the absence of added heparin because of the relative lack of mitogenic activity of wild-type human recombinant HBGF-1 in the absence of heparin. The possibility that the reduced mitogenic activity of the mutant HBGF-1 is related directly to its reduced apparent affinity for immobilized heparin is supported by the observation that the difference in the mitogenic potency between the wild-type and mutant protein is reduced to ~18-fold in the presence of 50 U/ml heparin.

In the second mitogenesis assay the abilities of the wild-type and mutant proteins to support the proliferation of human umbilical vein endothelial cells were compared. The results shown in Fig. 2 are consistent with those described above in that they demonstrate a dramatic heparin requirement of the wild-type HBGF-1 for biological activity and that the mutant HBGF-1 is not able to support cell proliferation to the same extent as the wild-type protein. These experiments were conducted in the presence of 50 U/ml heparin and the endothelial cells were seeded in the presence of 10 ng/ml wild-type HBGF-1. When growth assays were conducted in the presence of 5 U/ml heparin without wild-type protein during the seeding, mitogenic deficiencies of the mutant protein were more pronounced (Table I). The results shown in Fig. 3 demonstrate that the reduced mitogenic activity of the mutant HBGF-1 does not appear to be the result of any increased susceptibility of the protein to proteolytic digestion by components in serum or the conditioned media of NIH 3T3 cells.

Receptor-binding Activity of HBGF-1 Mutant p132E

The results presented above are consistent with the observations of Harper and Lobb (19) using bovine brain–derived HBGF-1 selectively methylated at lysine 132, although the magnitude of the reduction in mitogenic potency (~30-fold for 3T3 cell assay) as compared with the ~4-fold decrease reported by Harper and Lobb (19) is significantly greater. They also reported reduced receptor-binding activity for the modified protein. We examined the abilities of the wild-type and mutant recombinant HBGF-1 to compete with ^{125}I-labeled bovine HBGF-1 for binding to cell surface receptors on NIH 3T3 cells at a concentration of added heparin (5 U/ml) where the difference in mitogenic potencies of the two proteins was greatest.

The receptor-binding activity of the mutant HBGF-1 was established by competition for cross-linking of ^{125}I-HBGF-1 to 150,000- and 130,000-M_r proteins present on the surface of NIH 3T3 cells (16). The results shown in Fig. 4 demonstrate that the mutant HBGF-1 is similar to wild-type protein in its ability to compete for receptor–ligand cross-linking.

The functional consequences of HBGF-1 binding to its cell surface receptor include stimulation of protein tyrosine kinase activity (8, 15, 20) including phosphorylation of phospholipase C-γ (6). Fig. 5 *A* demonstrates that both wild-type and mutant HBGF-1 are able to increase the phosphotyrosine content of 150,000-, 90,000-, and 70,000-M_r proteins and, to a lesser extent, proteins with lower relative molecular masses as judged by Western blot analysis with phosphotyrosine-specific antibodies. The dose response and extent of activation is similar for the two forms of the growth factor. Stimulation of the phosphotyrosine content of phospholipase C-γ was examined by anti-phosphotyrosine Western blot analysis of 3T3 cell lysates after immunoprecipitation using antibodies that recognize phospholipase C-γ. Fig. 5 *B* demonstrates that mutant HBGF-1 shares with wild-type HBGF-1 the ability to stimulate tyrosine phosphorylation of phospholipase C-γ. These data regarding stimulation of tyrosine kinase activity by wild-type and mutant HBGF-1 are in good agreement with the receptor-binding data described above but do not provide insight into the functional basis for the relatively poor mitogenic capacity of this HBGF-1 mutant.

Protooncogene Induction by Wild-Type and Mutant HBGF-1

The results described above indicate that the functional properties of the mutant HBGF-1 associated with events that occur at the cell surface (i.e., receptor-binding and tyrosine kinase activation) are normal with respect to those of wild-type HBGF-1. In addition to tyrosine kinase activation, another early response to HBGF-1 receptor-binding is the elevation of protooncogene mRNA levels (17). To determine the effect of wild-type and mutant HBGF-1 on protooncogene expression, NIH 3T3 cells were serum starved and then either left unstimulated or stimulated with 10 ng/ml wild-type or mutant HBGF-1. Heparin (5 U/ml) was also added to the cells receiving growth factor. Cells were collected at various times after stimulation, RNA was prepared, and levels of c-*fos*, c-*jun*, c-*myc*, and glyceraldehyde 3-phosphate dehydrogenase mRNA (as a control for the amount of RNA loaded in each lane) were assayed by RNA gel blot analysis. Wild-type and mutant HBGF-1 increased protooncogene mRNA levels to a similar degree; maximal levels were observed at 30 min (c-*fos*, c-*jun*) or 2 h (c-*myc*) after stimulation (Fig.

Figure 3. Analysis of the relative stability of wild-type and mutant HBGF-1 in NIH 3T3 cell-conditioned media. The wild-type and mutant proteins were labeled and purified as described in Materials and Methods. The proteins were incubated in the presence of NIH 3T3 cell-conditioned media for 48 h at 37°C and then subjected to SDS-PAGE. The gels were dried and labeled proteins visualized by autoradiography. Lane 1 contains wild-type HBGF-1 and lane 2 mutant HBGF-1. The apparent molecular weights of both proteins are identical to that of HBGF-1 before incubation.

1 2

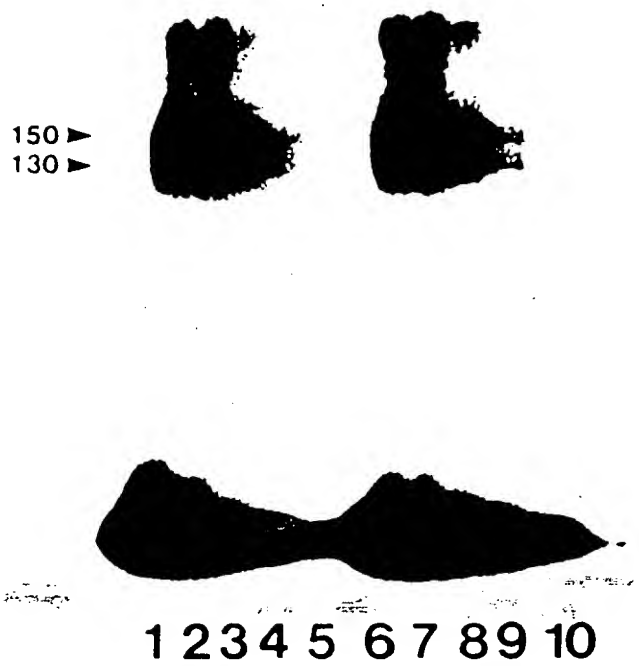


Figure 4. Ability of wild-type and mutant HBGF-1 to compete with ^{125}I-labeled bovine HBGF-1 for cross-linking to 150,000- and 130,000-mol wt cell surface receptors. NIH 3T3 cells were incubated with 1 ng/ml bovine ^{125}I-HBGF-1 and either 0.5, 1.0, 5.0, 10.0, or 50.0 ng/ml of wild-type (lanes 1–5) or mutant (lanes 6–10) human recombinant HBGF-1 in the presence of 5 U/ml heparin. After incubation, the cells were treated with cross-linking reagents as described in Materials and Methods. The apparent molecular weights of cross-linked species were determined after SDS-PAGE and autoradiography. The positions of two cross-linked 150,000- and 130,000-mol wt species, which correspond to the known apparent molecular weights of HBGF receptors, are indicated with arrows.

6). The addition of heparin alone did not induce protooncogene expression. Since the mitogenic differences between the wild-type and mutant HBGF-1 are more pronounced at lower growth factor concentrations, we also stimulated cells with 0.5, 1.0, 5.0, and 10 ng/ml wild-type and mutant growth factor (again in the presence of heparin). At all four concentrations used, the wild-type and mutant HBGF-1 were similar in their ability to induce c-*fos* mRNA expression (Fig. 7).

Overexpression of Wild-Type and Mutant HBGF-1 in Transfected NIH 3T3 Cells

It was demonstrated previously that overexpression of wild-type HBGF-1 in transfected Swiss 3T3 cells resulted in cells with an elongated, transformed morphological phenotype that grew to higher saturation densities (23). This transformed phenotype occurred even though the HBGF-1 polypeptide was not detectable in the conditioned media of these cells. We have shown that the mutant HBGF-1 is not a potent mitogen although it can bind receptor and initiate early events associated with mitogenic signal transduction. To investigate whether the intracellular function of the mutant HBGF-1 was altered, we examined the ability of this protein to induce a transformed phenotype in NIH 3T3 cells. Cells were either transfected with a plasmid conferring neomycin resistance or co-transfected with the neomycin resistance plasmid and wild-type or mutant HBGF-1 expression vectors.

Fig. 8 shows the results of Western blot analysis of transfected cell lysates using HBGF-1-specific antibodies. The Western blot analysis was normalized to cell number and provides the basis for our designation of relatively high or low levels of HBGF-1 expression. The results shown in Fig. 9 demonstrate that cells expressing a high level of wild-type HBGF-1 (Fig. 9 *B*) and to some extent a low level of wild-

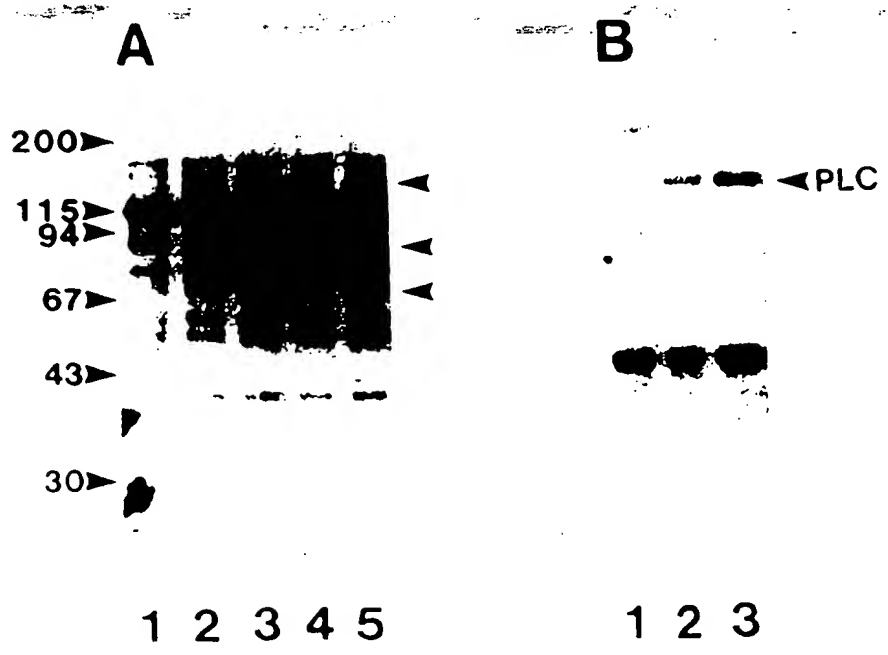


Figure 5. Stimulation of protein tyrosine kinase activity by wild-type and mutant HBGF-1. (*A*) Serum starved NIH 3T3 cells were either (lane *1*) unstimulated or treated with 5 U/ml heparin and (lane 2) 1 ng/ml wild-type; (lane *3*) 10 ng/ml wild-type; (lane *4*) 1 ng/ml mutant; or (lane *5*) 10 ng/ml mutant HBGF-1. The cells were processed as described in Materials and Methods and phosphotyrosine-containing proteins were visualized using antiphosphotyrosine antibodies and ^{125}I-protein A. The arrows indicate the positions of 150,000–, 90,000–, and 70,000–mol wt proteins whose phosphotyrosine content are increased by the addition of wild-type or mutant HBGF-1. (*B*) Cells were incubated as in *A* with the exception that cell lysates were immunoprecipitated with anti-phospholipase C-γ antibodies before Western blot analysis with anti-phosphotyrosine antibodies. Cells were either (lane *1*) unstimulated or treated with (lane *2*) 10 ng/ml wild-type, or (lane *3*) 10 ng/ml mutant HBGF-1. The arrow shows the position of a 150,000–mol wt protein whose phosphotyrosine content is increased by treatment with wild-type or mutant HBGF-1.

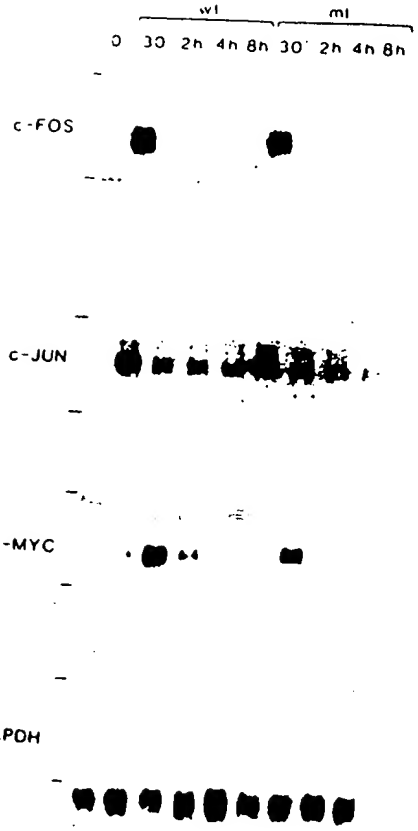


Figure 6. Effect of wild-type and mutant HBGF-1 on protooncogene mRNA levels. Serum starved NIH 3T3 cells were either left unstimulated or treated with heparin (5 U/ml) and 10 ng/ml wild-type (*wt*) or mutant (*mt*) HBGF-1 for the indicated time periods. RNA was prepared and used for RNA gel blot hybridization using the radiolabeled DNA probes indicated on the left side (*GAPDH*, glyceraldehyde 3-phosphate dehydrogenase). The upper and lower tick marks on the left side of each panel represent the positions of 28 and 18S rRNA, respectively.

type HBGF-1 (Fig. 9 *D*) have acquired a more polar, elongated phenotype characteristic of transformed 3T3 cells. This phenotype is not seen in cells expressing neomycin resistance alone (Fig. 9 *A*) or in cells expressing relatively high levels of mutant HBGF-1 (Fig. 9 *C*). It should be noted that we have not been able to detect HBGF-1 immunoreactivity in the media conditioned by these cells and that the cells expressing relatively high levels of wild-type HBGF-1 show enhanced growth in soft agar relative to untransfected cells or cells expressing high levels of the mutant HBGF-1 (data not shown). These results are consistent with the results of the mitogenic assays described above which demonstrate that the growth-promoting activity of the mutant HBGF-1 is relatively low when compared to the wild-type protein.

Discussion

The experiments described in this report were initiated as a result of the chemical modification studies of HBGF-1 reported by Harper and Lobb (19). They demonstrated that reductive methylation of HBGF-1 resulted in selective

stoichiometric modification of lysine residue 132 (using 1–154 numbering system for full-length HBGF-1). It was suggested that modification of this residue, which is conserved in all HBGF-1 and HBGF-2 sequences reported to date, is responsible for the reduced apparent affinity for immobilized heparin, the reduced mitogenic capacity, and the reduced receptor-binding activity of the modified protein. The results presented here using site-directed mutagenesis to address the role of lysine 132 on the functional properties of HBGF-1 are in general agreement with the conclusions of Harper and Lobb (19). Specifically, substitution of lysine 132 for glutamic acid reduces the apparent affinity of the recombinant protein for immobilized heparin (elutes at 0.45 M NaCl compared with 1.1 M NaCl for wild-type) and significantly reduces the mitogenic potency of the growth factor. The reduced mitogenic potency may be a direct consequence of the reduced apparent affinity of the mutant HBGF-1 for heparin since it has been demonstrated that the class 1 heparin binding growth factors in general (29) and human HBGF-1 in particular (22, 43) are dependent on the presence of heparin for optimal biological activity.

Our results do not support the notion that the reduced mitogenic capacity of HBGF-1 containing glutamic acid in place of lysine at position 132 is due to reduced binding to cell surface receptors. The receptor-binding properties of the mutant HBGF-1 are not distinguishable from those of the wild-type protein as judged by cross-linking experiments (see Fig. 4). In addition, the mutant HBGF-1 is able to induce the same pattern of tyrosine kinase phosphorylation as is the wild-type protein (see Fig. 5) and can induce protooncogene expression (see Fig. 6). The majority of the studies presented here utilize a heparin concentration of 5 U/ml; the concentration where maximal difference between the mitogenic activity of wild-type and mutant HBGF-1 was observed in the 3T3 cell thymidine incorporation assay. It should be noted that in the absence of heparin, the mutant HBGF-1 competes poorly with labeled wild-type HBGF-1 in cross-

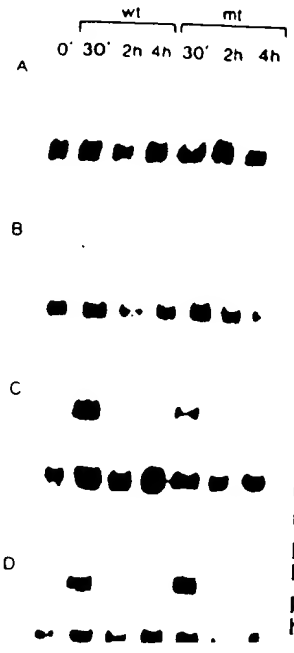


Figure 7. Effect of different concentrations of wild-type and mutant HBGF-1 on c-*fos* mRNA levels. Serum starved NIH 3T3 cells were either left unstimulated or treated with heparin (5 U/ml) and (*A*) 0.5 ng/ml, (*B*) 1.0 ng/ml, (*C*) 5.0 ng/ml, (*D*) 10 ng/ml wild-type (*wt*) or mutant (*mt*) HBGF-1 for the indicated time periods. RNA was prepared and used for RNA gel blot hybridization using the c-*fos* DNA probe (*upper panels*) or glyceraldehyde 3-phosphate dehydrogenase DNA

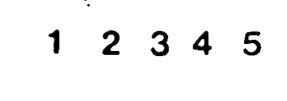


Figure Western blot analysis of HBGF-... NIH 3T3 cells transfected with wild-type or mutant HBGF-1 expression plasmids. NIH 3T3 cells were transfected as described in Materials and Methods. The figure shows the relative levels of HBGF-1 immunoreactivity present in lysates of cells transfected with wild-type HBGF-1 (lane *1*, clone producing relatively high level of HBGF-1; lane *3*, clone producing relatively low level of HBGF-1) normal NIH 3T3 cells (lane *2*), cells transfected with pSV2neo alone (lane *4*), and cells transfected with mutant HBGF-1 (lane *5*). For each cell type, 10^6 cells were lysed with 1 ml of 2× Laemmli sample buffer and a 60-μl aliquot was used in the Western blot.

linking assays (data not shown). In addition, whereas the apparent affinity of the mutant HBGF-1 for immobilized heparin is reduced, it does bind at ionic strengths (i.e., ~0.5 M NaCl) that exceed those known to be physiologic. Thus, the data presented here indicate that the mutant can utilize the presence of heparin to restore some (i.e., receptor-binding, tyrosine kinase activation, and protooncogene induction) but not all (i.e., stimulation of [^{3}H]thymidine incorporation into DNA and endothelial cell proliferation) of the activities of the wild-type protein. Similarly, it is of interest that the wild-type protein competes with labeled HBGF-1 for receptor-binding and induces protooncogene expression at similar concentrations in the presence or absence of added heparin yet it requires added heparin in order to promote DNA synthesis and cell proliferation (Figs. 1, 2, 4, and 6; and data not shown). Thus, the relatively poor mitogenic activity of the mutant protein may be related to its reduced apparent affinity for heparin. The data presented here demonstrate that "high" affinity receptor-binding, activation of tyrosine kinase activity, tyrosine phosphorylation of specific substrates, and induction of protooncogene expression may be necessary but are not, by themselves, sufficient to sustain a mitogenic response to the presence of HBGF-1. These results are consistent with the observations of Escobedo and Williams (12) who showed by site-directed mutagenesis of the PDGF receptor and cDNA-transfection that mutants could be constructed that were responsive to PDGF with respect to receptor tyrosine kinase activation and increased phosphatidylinositol turnover but did not elicit a mitogenic re-

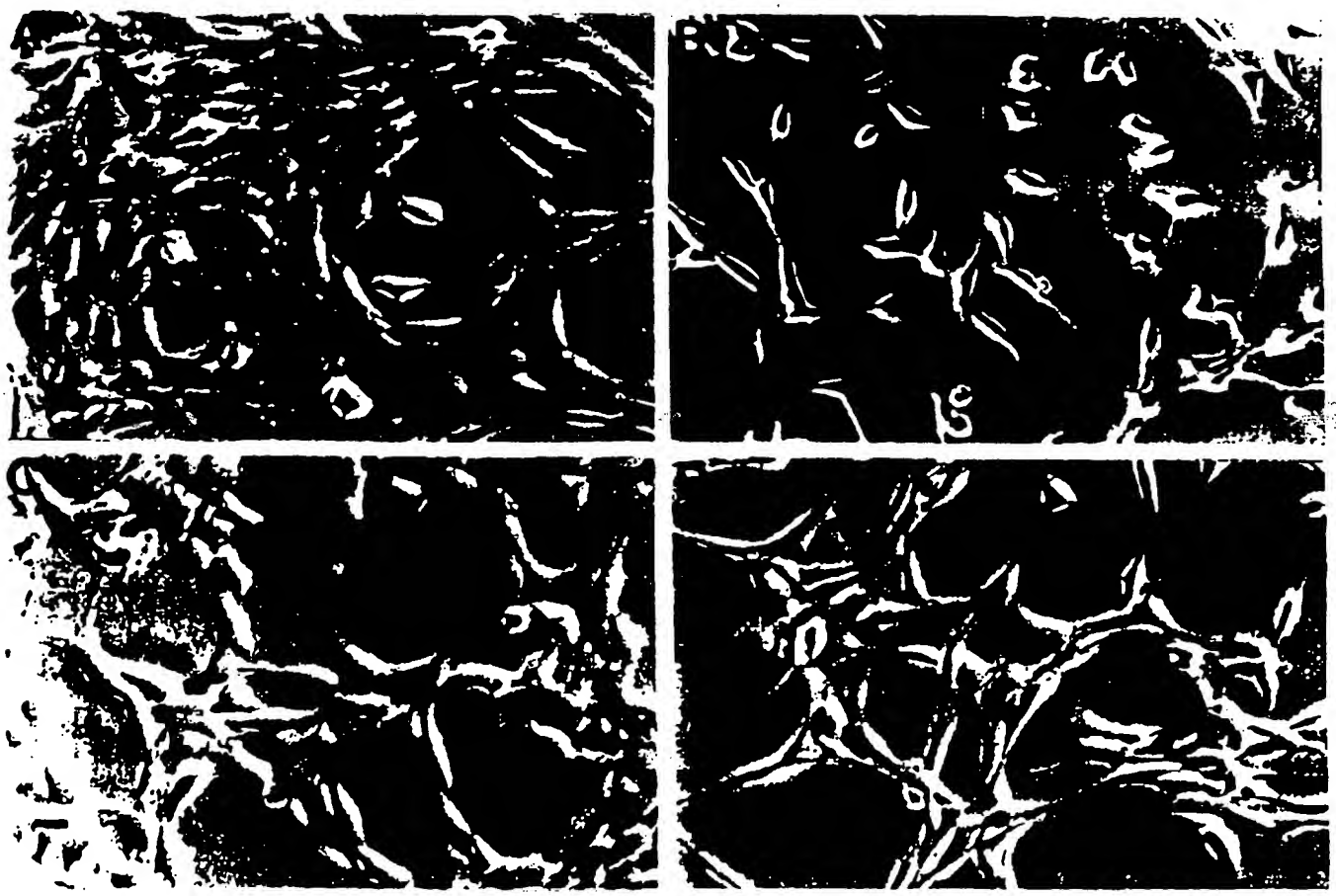


Figure 9. Morphology of NIH 3T3 cells transfected with wild-type or mutant HBGF-1 expression plasmids. The figure shows micrographs of the same NIH 3T3 cells analyzed by Western blot analysis in Fig. 8. *A* shows cells transfected with pSV2neo only and B–D show cells co-transfected with pSV2neo and expression vectors for wild-type (*B* and *D*) and mutant (*C*) HBGF-1. The cells shown in *B* correspond to those expressing relatively high levels of HBGF-1 (Fig. 8, lane *1*), whereas those shown in *D* correspond to those expressing relatively little HBGF-1 (Fig. 8, lane *3*).

sponse to PDGF. Similarly, Severinsson et al. (40) used similar methods to generate a system where the mutant receptor could mediate an increase in c-*fos* expression in response to PDGF but not actin reorganization or mitogenesis.

The mitogenic deficiencies of the mutant HBGF-1 may be due to reduced biological stability in tissue culture medium, reduced binding to cell surface proteoglycans, an altered intracellular stability, and/or an altered affinity for an intracellular receptor or binding protein. It has been established that the presence of heparin protects HBGF-1 from thermal and proteolytic inactivation (28, 37). In addition, it has been shown that ^{125}I-labeled HBGF-1 is relatively insensitive to lysosomal degradation after receptor-mediated endocytosis (14). There is no obvious difference in the susceptibility of wild-type and mutant HBGF-1 to proteolytic cleavage by the conditioned media of NIH 3T3 cells cultured in the presence of 10% calf serum. However, the relative resistance of wild-type and mutant HBGF-1 to proteolytic modification in the presence of target cells or after receptor-mediated endocytosis has not been established. It is also possible that the mutant protein is more susceptible than the wild type to nonproteolytic inactivation. Further studies should reveal whether the altered activities of the mutant HBGF-1 are a consequence of its reduced apparent affinity for heparin.

In summary, the data presented here demonstrate that the various functions of HBGF-1 can be dissociated at the structural level. The observation that site-directed mutagenesis can be used to produce recombinant proteins with "normal" receptor-binding activity and reduced mitogenic activity indicates that similar methods could be used to produce potent antagonists of HBGF-1. More importantly, these results indicate that it may be possible through structure-function analysis and site-directed mutagenesis to generate mutants that retain certain (i.e., chemotactic, mitogenic, or heparin-binding) but not other biological functions characteristic of the wild-type protein. Finally, whereas the data presented on the receptor-binding and tyrosine kinase activation properties of the p132E mutant demonstrate that a lysine residue at this position is not critical for these functions, it is still possible that methylation of a lysine at this position could lead to reduced receptor-binding activity of HBGF-1 (19).

The authors thank Tevie Mehlman and M. Leslie O'Connor for their help in the early stages of this work, and Sally Young for her help in the preparation of the manuscript. Portions of this work were done by P. J. Donohue in partial fulfillment of the Ph.D. requirements in the Graduate Genetics Program at George Washington University, Washington, D.C.

This work was supported in part by National Institutes of Health grants HL 35762 to Wilson H. Burgess and HL 39727 to Jeffrey A. Winkles, and a grant-in-aid from the American Heart Association (891047) to Wilson H. Burgess, with funds contributed in part by the American Heart Association Maryland Affiliate, Inc.

Received for publication 15 February 1990 and in revised form 23 July 1990

References

1. Baird, A., D. Schubert, N. Ling, and R. Guillemin. 1986. Receptor- and heparin-binding domains of basic fibroblast growth factor. *Proc. Natl. Acad. Sci. USA.* 85:2324–2328.
2. Bouche, G., N. Gas, H. Prats, V. Baldin, J.-P. Tauber, J. Teissié, and F. Amalric. 1987. Basic fibroblast growth factor enters the nucleolus and stimulates the transcription of ribosomal genes in ABAE cells undergoing $G_0 \rightarrow G_1$ transition. *Proc. Natl. Acad. Sci. USA.* 84:6770–6774.
3. Burgess, W. H., and T. Maciag. 1989. The heparin-binding (fibroblast growth factor family of proteins. *Annu. Rev. Bio*
4. Burgess, W. H., T. Mehlman, R. Friesel, W. V. Jo 1985. Multiple forms of endothelial cell growth fac biological and chemical characterization. *J. Bio* 11392.
5. Burgess, W. H., T. Mehlman, D. R. Marshak, Maciag. 1986. Structural evidence that endothelia is the precursor of both endothelial cell growth fac blast growth factor. *Proc. Natl. Acad. Sci. USA.*
6. Burgess, W. H., C. A. Dionne, U. Kaplow, R. Mud berstein, J. Schlessinger, and M. Jaye. 1990. cDNA cloning of phospholipase c–a major substra growth factor 1 (acidic fibroblast growth factor)–a nase. *Mol. Cell Biol.* 10:4770–4777.
7. Casey, G., R. Smith, D. McGillivray, G. Peters, an Characterization and chromosome assignment of th int-2, a potential proto-oncogene. *Mol. Cell. Biol.*
8. Coughlin, S. R., J. Barr, L. S. Coussens, L. J. Fretto, 1988. Acidic and basic fibroblast growth factors stim activity *in vivo*. *J. Biol. Chem.* 263:988–993.
9. Delli Bovi, P., and C. Basilico. 1987. Isolation of a transforming gene following transfection of Kaposi sa *Natl. Acad. Sci. USA.* 84:5660–5664.
10. Dickson, C., and G. Peters. 1987. Potential oncogene growth factors. *Nature (Lond.).* 326:833–836.
11. Dickson, C., R. Smith, S. Brookes, and G. Peters. 19 by mouse mammary tumor virus: proviral activation in the common integration region int-2. *Cell.* 37:525
12. Escobedo, J. A., and L. T. Williams. 1988. A PDGF re sential for mitogenesis but not for many other response *(Lond.).* 355:85–87.
13. Finch, P. W., J. S. Rubin, T. Miki, D. Ron, and S. Aar man KGF is FGF-related with properties of a paracrine lial cell growth. *Science (Wash. DC).* 245:752–755.
14. Friesel, R., and T. Maciag. 1988. Internalization an heparin-binding growth factor-1 by endothelial cells. *B Res. Commun.* 151:957–964.
15. Friesel, R., W. H. Burgess, and T. Maciag. 1989. Heparin factor 1 stimulates tyrosine phosphorylation in NIH 3T *Biol.* 9:1857–1865.
16. Friesel, R., W. H. Burgess, T. Mehlman, and T. Maciag. actenzation of the receptor for endothelial cell growth f ligand attachment. *J. Biol. Chem.* 261:7581–7584.
17. Gay, C. G., and J. A. Winkles. 1990. Heparin-binding stimulation of human endothelial cells induces platelet factor A-chain gene expression. *J. Biol. Chem.* 265:32
18. Gospodarowicz, D., G. Neufeld, and L. Schweigerer. growth factor: structural and biological properties. *J* 5:15–26.
19. Harper, J. W., and R. R. Lobb. 1988. Reductive methy residues in acidic fibroblast growth factor: effect on m and heparin affinity. *Biochemistry.* 27:671–678.
20. Huang, S. S., and J. S. Huang. 1986. Association of bovi growth factor receptor with protein tyrosine kinase a *Chem.* 261:9568–9571.
21. Jaye, M., R. Howk, W. Burgess, G. A. Ricca, I. Chier, S. J. O'Brien, W. S. Modi, T. Maciag, and W. N. Drohar endothelial cell growth factor: cloning, nucleotide sequen some localization. *Science (Wash. DC).* 233:541–545.
22. Jaye, M., W. H. Burgess, A. B. Shaw, and W. N. Drohan. equivalence of natural bovine and recombinant human α growth factor. *J. Biol. Chem.* 262:16612–16617.
23. Jaye, M., R. M. Lyall, R. Mudd, J. Schlessinger, and N. Expression of acidic fibroblast growth factor cDNA conf vantage and tumorigenesis in Swiss 3T3 cells. *EMBO (E Organ.) J.* 7:963–969.
24. Kan, M., D. DiSorbo, J. Hou, H. Hoshi, P. E. Mansso McKeehan. 1988. High and low affinity binding of h growth factor to a 130-kDa receptor correlates with stimul bition of growth of a differentiated human hepatoma cell. 263:11306–11313.
25. Koda, T., A. Sasaki, S. Matsushima, and M. Kakinama. forming gene, hst, found in NIH 3T3 cells transformed w stomach cancers and a colon cancer. *Jpn. J. Cancer Res*
26. Laemmli, U. K. 1970. Cleavage of structural proteins durin of the head of bacteriophage T4. *Nature (Lond.).* 277:6
27. Lobb, R. R. 1988. Clinical applications of heparin-binding g *Eur. J. Clin. Invest.* 18:321–336.
28. Lobb, R. R. 1988. Thrombin inactivates acidic fibroblast gro not basic fibroblast growth factor. *Biochemistry.* 27:2572
29. Lobb, R. R., J. W. Harper, and J. W. Fett. 1986. Purificati binding growth factors. *Anal. Biochem.* 154:1–14.
30. Deleted in proof.
31. Margolis, B., S. G. Rhee, S. Felder, M. Mervic, R. Lyall, A. Ullrich, A. Zilberstein, and J. Schlessinger. 1989. EGF

EGF receptor signaling. *Cell.* 57:1101–1107.

32. Marics, I., J. Adelaide, F. Raybaud, M.-G. Mattei, F. Coulier, J. Planche, O. Rapeynere, and D. Birnbaum. 1989. Characterization of the HST-related FGF.6 gene, a new member of the fibroblast growth factor gene family. *Oncogene.* 4:335–340.
33. Mehlman, T., and W. H. Burgess. 1990. Identification and characterization of heparin binding proteins using a gel overlay procedure. *Anal. Biochem.* 188:159–163.
34. Moscatelli, D. 1988. Metabolism of receptor-bound and matrix-bound basic fibroblast growth factor by bovine capillary endothelial cells. *J. Cell Biol.* 107:753–759.
35. Renko, M., N.Quarto, T. Morimoto, and D. B. Rifkin. 1990. Nuclear and cytoplasmic localization of different basic fibroblast growth factor species. *J. Cell. Physiol.* 144:108–114.
36. Rifkin, D. B., and D. Moscatelli. 1989. Recent developments in the cell biology of basic fibroblast growth factor. *J. Cell. Biol.* 109:1–6.
37. Rosengart, T. K., W. Johnson, R. Friesel, R. Clark, and T. Maciag. 1988. Heparin protects heparin-binding growth factor-1 from proteolytic inactivation *in vitro*. *Biochem. Biophys. Res. Commun.* 152:432–440.
38. Sakamoto, H., M. Mori, M. Taira, T. Yoshida, S. Matsukawa, K. Shimizu, M. Terada, and T. Sugimura. 1986. Transforming gene from human stomach cancers and a noncancerous portion of stomach mucosa. *Proc. Natl. Acad. Sci. USA.* 83:3997–4001.
39. Schubert, D., N. Ling, and A. Baird. 1987. Multiple influences of a heparin-binding growth factor on neuronal development. *J. Cell Biol.* 104:635–643.
40. Severinsson, L., B. Ek, K. Mellstrom, L. Claesson-Welsh, and C.-H. Heldin. 1990. Deletion of the kinase insert sequence of the platelet-derived growth factor β-receptor affects receptor kinase activity and signal transduction. *Mol. Cell. Biol.* 10:801–809.
41. Southern, P. J., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.* 1:327–341.
42. Ueno, N., A. Baird, F. Esch, N. Ling, and R. Guillemin. 1986. Isolation of an amino terminal extended form of basic fibroblast growth factor. *Biochem. Biophys. Res. Comm.* 138:580–588.
43. Uhlrich, S., D. Lagente, J. Choay, Y. Courtois, and M. Lenfant. 1986. Structure activity relationship in heparin: stimulation of non-vascular cells by a synthetic heparin pentasaccharide in cooperation with human acidic fibroblast growth factors. *Biochem. Biophys. Res. Commun.* 139:728–732.
44. Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin. 1979. DNA-mediated transfer of the adenine phosphoribosyl transferase locus into mammalian cells. *Proc. Natl. Acad. Sci. USA.* 76:1373–1376.
45. Yuasa, Y., and K. Sudo. 1987. Transforming genes in human hepatomas detected with a tumorigenicity assay. *Jpn. J. Cancer Res.* 78:1036–1040.
46. Zhan, X., A. Culpepper, M. Reddy, J. Loveless, and M. Goldfarb. 1987. Human oncogenes detected by a defined medium culture assay. *Oncogene.* 1:369–376.
47. Zhan, X., B. Bates, X. Hu, and M. Goldfarb. 1988. The human FGF-5 oncogene encodes a novel protein related to fibroblast growth factors. *Mol. Cell. Biol.* 8:3487–3495.

1
Characteristics of the amino acids as components of a peptide hormone sequence

J. Rudinger (Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule, Zürich, Switzerland)

In the living organism the polypeptide chains of proteins are used for the most diverse purposes: structural support and protection, catalysis of a wide range of chemical reactions, energy transduction, food storage, transport, and—among many others—regulation and co-ordination by information transfer (including humoral transmission by hormones). Molecules with the varied properties required for these multifarious functions are built up of a mere 20 units: the protein-constituent amino acids, connected primarily in a single structural mode (a linear sequence) but finally arranged in space in intricate ways to molecules of the required size, shape and properties. Although the peptide hormones fulfil such a highly specialised role, there is nothing to distinguish, *a priori*, their sequences from other polypeptide or protein sequences with different biological functions, or no function at all.

THE AMINO ACIDS

The 'proteinogenic' amino acids, though few in number, exhibit between them a remarkable range of chemical, physical and steric features. They are arranged in figure 1.1 in such a way that lines can be drawn to indicate their classification according to various properties. For instance, the sidechains may be hydrophilic (outside the hook-shaped line) or hydrophobic (within the hook); glycine, lying on the line, is taken as the reference amino acid for this purpose.

Again, some sidechains are chemically inert (those to the left of the broken line), while those to the right show varying kinds and degrees of chemical reactivity and may be capable of substitution, hydrogen bond or salt formation, oxidation, etc. Other classifications are shown by the use of frames. Some of the reactive sidechains are neutral, but others (shown by the lower right-hand frame) are charged either positively or negatively in the physio-

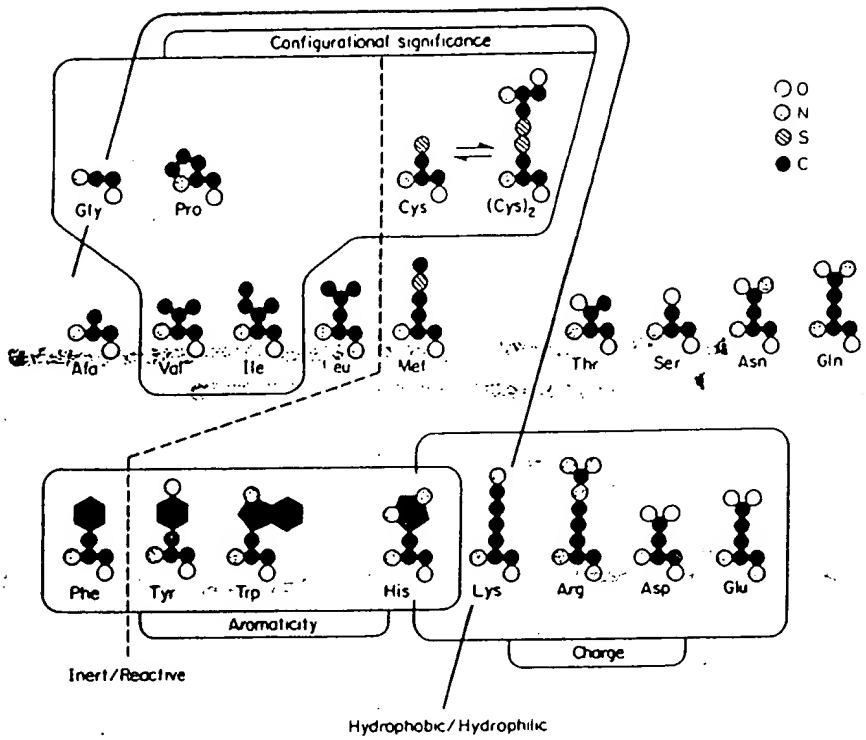


FIGURE 1.1 Schematic representation of the 20 amino acyl residues found in proteins, and their classification by certain properties; for details see text

logical pH range. Histidine may or may not be protonated under physiological conditions. The two basic amino acids (lysine and arginine) differ in their equilibrium constants (arginine being the stronger base) and so do the two acidic amino acids (aspartic being a somewhat stronger acid than glutamic). Thus, between them, the charged amino acids encompass a wide range of pK values.

The lower left-hand frame encloses those sidechains which contain aromatic structures and exhibit corresponding special properties (π-electron interactions). Finally, the frame at the top draws attention to amino acids with special steric properties which affect the way in which the peptide chain can be arranged in three dimensions (its conformation or secondary structure). Glycine, with no obtruding sidechain, offers a particularly high degree of conformational freedom, whereas the bulky, β-branched sidechains of valine and isoleucine severely restrict the way in which the peptide chain may fold. A still greater degree of constraint is imposed by the rigid cyclic structure of proline. Proline has also the special property that the peptide bond in which its imino group participates may have either the *cis* or the *trans* conformation, whereas all other peptide bonds are normally

confined to the *trans* geomet
cysteine sidechains provides
linking and thereby stabi
peptide chains.

It will be noted that most
fying 'boxes'—in other worc
features which may be utili
taneously. As a result, it is i
residue in a sequence. A giv
same 'significance' in diffe
positions of the same sequer

For instance, isoleucine is
and in position 5 of angiote
may be replaced, without a
β-branched amino acids pr
the steric requirements are
the diastereomeric alloisolet
1972; Jorgensen and Weink

Another illustration that
provided by the molecule of
proline in position 3 by alani
assayed on the rabbit blood p
of proline are of no significan
substitution of the proline in
and in position 7 to 0.1 per c
sarcosine (*N*-methylglycine)
these sites, the resulting ana
alanine derivatives (they re
activity of the parent compo

We may therefore conclu
sequence it is the *N*-alkylat

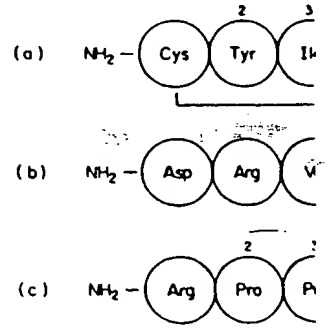


FIGURE 1.2 Sequences of

confined to the *trans* geometry. The formation of disulphide bridges between cysteine sidechains provides an even more positive way of covalently cross-linking and thereby stabilising the three-dimensional arrangement of peptide chains.

It will be noted that most amino acids occur in two or more of the classifying 'boxes'—in other words, each generally has several different structural features which may be utilised in protein building alternatively or simultaneously. As a result, it is impossible to attach a unique significance to any residue in a sequence. A given amino acid will not by any means have the same 'significance' in different peptide sequences, or even in different positions of the same sequence.

For instance, isoleucine is found both in position 3 of oxytocin (figure 1.2a) and in position 5 of angiotensin II (figure 1.2b). Whereas in angiotensin it may be replaced, without appreciable loss of biological activity, by other β-branched amino acids provided they are equally lipophilic, in oxytocin the steric requirements are much more stringent and even replacement by the diastereomeric alloisoleucine causes a drastic fall in activity (Rudinger, 1972; Jorgensen and Weinkam, 1973).

Another illustration that sidechain 'significance' depends on 'context' is provided by the molecule of bradykinin (figure 1. 2c). Replacement of the proline in position 3 by alanine does not affect the potency of the peptide as assayed on the rabbit blood pressure; obviously, the special steric properties of proline are of no significance in this position. On the other hand, the same substitution of the proline in position 2 reduces the activity to 0.5 per cent, and in position 7 to 0.1 per cent (Schröder and Hempel, 1964). Moreover, if sarcosine (*N*-methylglycine) rather than alanine is used to replace proline at these sites, the resulting analogues are considerably more active than the alanine derivatives (they retain, respectively, 50 and 30 per cent of the activity of the parent compound; Yanaihara *et al.*, 1966).

We may therefore conclude that in positions 2 and 7 of the bradykinin sequence it is the *N*-alkylation of the nitrogen in the peptide backbone

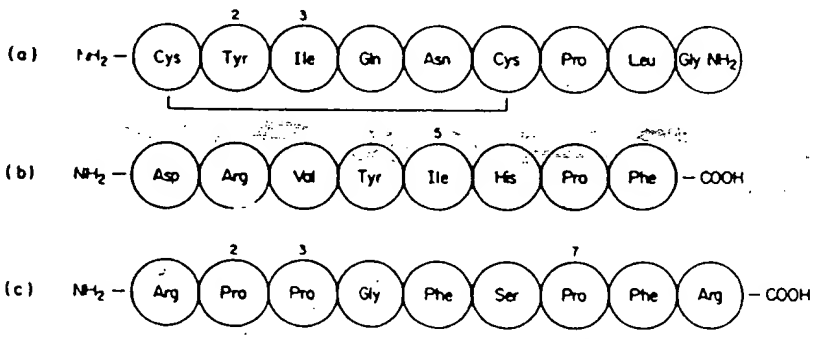


FIGURE 1.2 Sequences of oxytocin (a), angiotensin II (b) and bradykinin (c)

which is the important feature of the proline structure rather than, for example, the presence of its ring or its lipophilic properties.

SEQUENCE AND CONFORMATION

The humoral mechanism of information transfer requires that the effect of a hormone on its receptor be both sensitive and specific. These requirements can be met only by hormone–receptor binding based on multiple interactions of complementary sites: it is a *pattern* on the hormone molecule which is 'recognised' by the receptor in the binding process. Generally, parts of several amino acids of the sequence will participate in forming this pattern. In peptide molecules which are short or conformationally very flexible, or both, the pattern will often involve the sidechains of amino acids which are close together in the primary peptide sequence ('continuate' or 'synchnologic' read-out (Schwyzer, 1972), analogous to 'sequential' determinants in antigens (Sela, 1969)).

However, in peptides whose conformation is stabilised either by a sufficient number of intramolecular, non-covalent sidechain interactions or by disulphide bonds, or both, the critical topochemical pattern may be made up of groups widely separated in the linear sequence (Hofmann and Katsoyannis, 1963; Schwyzer, 1963; Rudinger and Jošt, 1964) ('discontinuate' or 'rhegnylogic' read-out, analogous to 'conformational' determinants).

Whereas in the first case the often-cited analogy to a 'message' written in linear, alphabetic script is valid, a topochemical arrangement of the second type is better likened to Chinese writing: it is the *pattern* of the character which conveys the meaning (figure 1.3) and not the (prescribed) order in which the brush strokes are made.

In either case it should be noted that the conformation of the hormone molecule in its interaction with the receptor need not be identical with its conformation in solution (cf. Rudinger and Jošt, 1964; Rudinger, 1972),

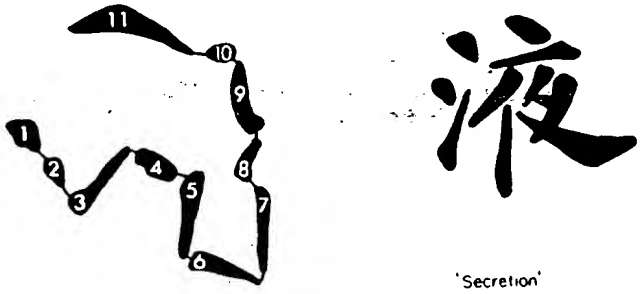


FIGURE 1.3 Eleven brush-strokes in the order in which they are made but in random pattern (left), and in the pattern in which they make up the Chinese character for 'secretion'

although major, energetically disfavou
admittedly unlikely.

SIGNIFIC

In a given molecule some amino acid
'significance' to their inclusion in the
in recognition by, and binding to, the
existence of this pattern is dependen
intramolecular interactions, as discuss
acids or sequences contributing to this
less 'significant' for the biological ac
generally, sequences contributing to th
affect its transport and distribution, m
non-receptor sites, etc., may signific
In defining the relation between sequer
into account all these contributions, a
in receptor binding*.

Two separate events may be conc
receptor interaction: binding (recogn
the signal which eventually leads to th
models proposed to account for hor
generation is actually identical with the
of the 'allosteric' model (Monod, Cha
common the assumption that bindin
activity of a second, topologically di
bably by inducing a conformational c
tion' type; Rudinger, Pliška and Kr
ration are two distinct molecular even

The properties of a series of oxyto
residue (position 2) illustrate a po
problem and favour a 'participatic
figure 1.4, replacement of the hydr
substituents leads, in a graded manne
the appearance of inhibitor properti
from pharmacological parameters (r
the whole series of analogues, sugg
around position 2 is involved in st
to binding (see Rudinger *et al.*, 1972
substitution *ortho* to the tyrosine hy
methyl—also gives rise to inhibi

*Obviously, these various functions need not
and distinct sequences, but particular amin
participate in several of them.

although major, energetically disfavoured structural rearrangements are admittedly unlikely.

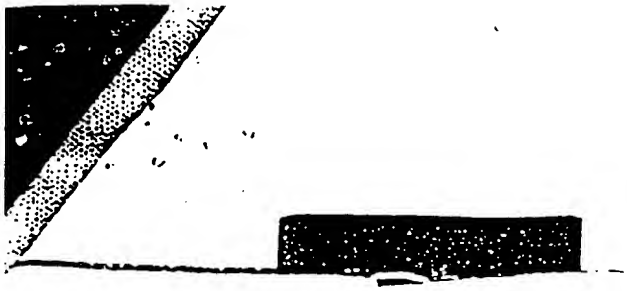
SIGNIFICANCE

In a given molecule some amino acids or sequences obviously owe their 'significance' to their inclusion in the pattern which is directly involved in recognition by, and binding to, the receptor. However, the fact that the existence of this pattern is dependent on a conformation stabilised by intramolecular interactions, as discussed above, implies that other amino acids or sequences contributing to this conformational stability will be no less 'significant' for the biological activity of the molecule. Even more generally, sequences contributing to those properties of the peptide which affect its transport and distribution, metabolic transformations, binding to non-receptor sites, etc., may significantly modify its biological activity. In defining the relation between sequence and activity it is necessary to take into account all these contributions, and not merely those directly involved in receptor binding*.

Two separate events may be conceptually distinguished in hormone–receptor interaction: binding (recognition) and stimulation (initiation of the signal which eventually leads to the observed response). In some of the models proposed to account for hormone action, the process of stimulus generation is actually identical with the process of binding. These are variants of the 'allosteric' model (Monod, Changeux and Jacob, 1963), which have in common the assumption that binding of hormone at one site modifies the activity of a second, topologically distinct site on the same molecule, probably by inducing a conformational change. In other models (the 'participation' type; Rudinger, Pliška and Krejčí, 1972) binding and stimulus generation are two distinct molecular events.

The properties of a series of oxytocin analogues modified at the tyrosine residue (position 2) illustrate a possible experimental approach to this problem and favour a 'participation' model for oxytocin. As shown in figure 1.4, replacement of the hydroxyl group of this tyrosine by various substituents leads, in a graded manner, to loss of oxytocin-like activity and to the appearance of inhibitor properties. Yet the binding affinity, determined from pharmacological parameters (pD_2 and pA_2), is practically the same for the whole series of analogues, suggesting that the region of the molecule around position 2 is involved in stimulus generation but contributes little to binding (see Rudinger *et al.*, 1972). Moreover, we have recently found that substitution *ortho* to the tyrosine hydroxyl group—for example, by iodine or methyl—also gives rise to inhibitors: evidently the hydroxyl group is

*Obviously, these various functions need not and, in general, will not be attributable to separate and distinct sequences, but particular amino acids, sequences or topochemical regions may participate in several of them.



r Control Mechanisms

BIOLOGICAL COUNCIL

The Co-ordinating Committee for Symposia
on Drug Action

PEPTIDE HORMONES

Edited by

J. A. PARSONS, M.A., B.M., B.Ch.

National Institute for Medical Research
Mill Hill, London

University Park Press
Baltimore London Tokyo

June 1976 (but now out of print)

according to University Park Press
(800) 638-7511

AO

MOLECULAR AND CELLULAR BIOLOGY, Mar. 1988, p. 1247–1252
0270-7306/88/031247-06$02.00/0

Vol. 8, No. 3

Transforming Growth Factor α: Mutation of Aspartic Acid 47 and Leucine 48 Results in Different Biological Activities

ELIANE LAZAR,† SHINICHI WATANABE,* STEPHEN DALTON, AND MICHAEL B. SPORN

Laboratory of Chemoprevention, National Cancer Institute, Bethesda, Maryland 20892

Received 22 July 1987/Accepted 30 November 1987

To study the relationship between the primary structure of transforming growth factor α (TGF-α) and some of its functional properties (competition with epidermal growth factor (EGF) for binding to the EGF receptor and induction of anchorage-independent growth), we introduced single amino acid mutations into the sequence for the fully processed, 50-amino-acid human TGF-α. The wild-type and mutant proteins were expressed in a vector by using a yeast α mating pheromone promoter. Mutations of two amino acids that are conserved in the family of the EGF-like peptides and are located in the carboxy-terminal part of TGF-α resulted in different biological effects. When aspartic acid 47 was mutated to alanine or asparagine, biological activity was retained; in contrast, substitutions of this residue with serine or glutamic acid generated mutants with reduced binding and colony-forming capacities. When leucine 48 was mutated to alanine, a complete loss of binding and colony-forming abilities resulted; mutation of leucine 48 to isoleucine or methionine resulted in very low activities. Our data suggest that these two adjacent conserved amino acids in positions 47 and 48 play different roles in defining the structure and/or biological activity of TGF-α and that the carboxy terminus of TGF-α is involved in interactions with cellular TGF-α receptors. The side chain of leucine 48 appears to be crucial either indirectly in determining the biologically active conformation of TGF-α or directly in the molecular recognition of TGF-α by its receptor.

Transforming growth factor α (TGF-α) is a polypeptide of amino acids. First isolated from a retrovirus-transformed ouse cell line (9), it has subsequently been found in human mor cells (10, 29), in the early rat embryo (18), and cently in cell cultures from the pituitary gland (23). TGF-α pears to be closely related to epidermal growth factor GF) structurally and functionally (19, 20). The two pep- es apparently bind to the same receptor, and both induce chorage-independent growth of certain nontransformed ells, such as NRK cells, in the presence of TGF-β (1).

Comparison of amino acid sequences reveals about 35% omology among the EGF-like peptides (rat [27], mouse 25], and human [13] EGFs and rat [19] and human [12] GF-αs). Some viral peptides (Shope fibroma growth factor 6], vaccinia growth factor [2], and myxoma growth factor 0]) also share homologies with the EGF-like peptides.

If TGF-α is involved in transformation, a TGF-α antago- st could be an important therapeutic tool in the treatment certain types of malignancies. An understanding of the nformational and dynamic properties of the TGF-α mole- ule is basic to the design of an antagonist. A hypothetical ntagonist would bind to the same receptor as TGF-α, but ould not induce the series of proliferative and transforming ents induced by TGF-α. To obtain such a molecule it is cessary to dissociate interactions responsible for binding om those involved in signal transduction. We decided to proach the problem by way of site-directed mutagenesis of human sequence of TGF-α. In this report we describe our t series of mutations, which were carried out at residues sp-47 and Leu-48, in the carboxy-terminal part of TGF-α: ese two amino acids are highly conserved in the EGF-like mily of peptides. We show that these two adjacent residues play different roles in the structure and/or function of TGF-α.

MATERIALS AND METHODS

Cells. Normal rat kidney (NRK) cells were grown in Dulbecco modified Eagle medium containing 10% (vol/vol) calf serum.

TGF-α gene. The sequence of the 50-amino-acid human TGF-α was originally derived from a human TGF-α precursor cDNA (12). The coding sequence is preceded by an ATG methionine codon and followed by a TAA stop codon and is flanked by *Eco*RI restriction sites. This *Eco*RI fragment combines the 59-base-pair *Eco*RI-*Nco*I fragment from plasmid pTE5 (12) with the 111-base-pair *Nco*I-*Eco*RI fragment from plasmid pyTE2 (11). The resulting *Eco*RI fragment was inserted in M13mp18 for site-directed mutagenesis.

Synthesis and purification of oligonucleotides and oligonucleotide-directed mutagenesis. The synthesis and purification of 20- to 27-nucleotide oligonucleotides were carried out as described previously (31). The one or two nucleotides responsible for the mutation were located in the middle of the oligonucleotide. Mutagenesis was performed by published procedures (21, 33). The sequences of the mutant clones were verified by the method of Sanger et al. (25).

Yeast shuttle vector. The vector YEp70αT contains a yeast α-factor pheromone promoter and prepro sequence for the expression of TGF-α (15). The mutant TGF-α coding sequence was inserted in the *Eco*RI site of plasmid YEp70αT and expressed in the form of a fusion protein consisting of 92 amino acids from the prepro sequence of the yeast α factor attached to the amino terminus of TGF-α (28). The yeast cleaves the precursor and secretes TGF-α with 8 amino acids fused to it (4 are encoded by the prepro sequence of α-factor, and the other 4 are encoded by the DNA sequence added to insert of the TGF-α gene). The last of these residues is a methionine, which allows the cleavage of the

* Corresponding author.
† Present address: Unité d'Oncologie Moleculaire, IRSC, 94802 ejuif Cedex, France.

protein by cyanogen bromide (CNBr) and the release of a mature TGF-α (50 amino acids) (see Results).

Yeast strain and transformation. The yeast *Saccharomyces cerevisiae* 20B-12 (*MATα trp1 pep4-3*) (17) was obtained from the Yeast Genetics Stock Center, Berkeley, Calif. *S. cerevisiae* 20B-12 was grown in YEPD medium (1% yeast extract [Difco Laboratories], 2% Bacto-Peptone [Difco], 2% glucose). When the culture reached an optical density at 660 nm of 1, spheroplasts were prepared (14) for transformation. For each transformation we used 10 to 15 μg of purified plasmid DNA.

Partial purification of TGF-α mutants. At 3 days after transformation, five individual colonies of transformants were grown to saturation in YEPD medium. The amount of protein in the yeast medium was measured by the method of Bradford (3), and the amount of mutant TGF-α secreted in the yeast medium was determined by radioimmunoassay. The clones which secrete the highest amount of mutant TGF-α were used to grow a 1-liter culture in YNB-CAA medium (0.67% yeast nitrogen base, 20 g of glucose per liter, 10 g of Casamino Acids [Difco] per liter). After the culture reached saturation (optical density at 660 nm of 10 to 12) (48 h in an air shaker at 30°C), the yeast conditioned medium was dialyzed extensively against 1 M acetic acid in 3,000-molecular-weight cutoff dialysis tubing. Usually 250 ml of dialyzed culture was lyophilized, suspended in 10 ml of 70% formic acid, and treated with CNBr (molar excess of 500) for 20 h at room temperature. The CNBr was subsequently evaporated, and the samples were lyophilized. CNBr-treated samples were suspended in 1 ml of 1 M acetic acid, loaded on a Bio-gel P30 column (30 by 1.5 cm [Bio-Rad Laboratories]), and eluted with 1 M acetic acid. Fractions of 1 ml were collected. Aliquots were lyophilized, suspended in binding buffer (minimum essential medium containing 1 mg of bovine serum albumin per ml and 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.4]), neutralized if necessary to pH 7.4, and tested in EGF-binding competition and soft-agar assays, as well in radioimmunoassay.

Radioimmunoassays. The amounts of TGF-α secreted in the yeast medium were determined by radioimmunoassay with the immunoglobulin G fraction of a polyclonal antibody, 34D, raised against recombinant human TGF-α (4), in 0.1 M Tris (pH 7.5)–0.15 M NaCl–2.5 mg of bovine serum albumin per ml. The amounts of partially purified TGF-α present in the P30 column fractions were measured by using the Biotope RIA kit with polyclonal antibody against human TGF-α (a gift from W. Hargreaves, Biotope), under denaturing conditions, as recommended by the supplier.

EGF binding competition assay and soft agar assay. Both EGF-binding competition and soft-agar assays have been described previously (1).

RESULTS

Rationale for mutations in the carboxyl terminus of TGF-α. Figure 1 shows the amino acid sequence of TGF-α in which the residues that are conserved among all the EGF-like peptides described thus far (EGF, TGF-α, and EGF-like viral proteins) are enclosed in bold circles. Among the 11 conserved amino acids, there are 6 Cys and 2 Gly residues, which presumably play essential roles in determining the overall conformation of the molecule. We concentrated on the two conserved amino acids in the carboxyl terminus, Asp-47 and Leu-48. The Asp in position 47 is conserved among the EGFs and TGF-α (human or murine), but not among the EGF-like viral proteins (vaccinia growth factor, Shope fibroma growth factor, or myxoma growth factor), whereas Leu 48 is conserved among all the EGF-like peptides so far described. In both mouse and human EGF, the two corresponding residues (Asp-46 and Leu-47) are located near the surface of the protein (8, 22, 22a). We designed a series of mutations in these two positions.

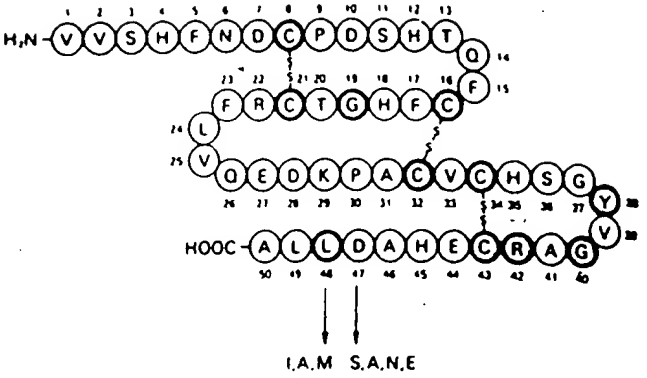


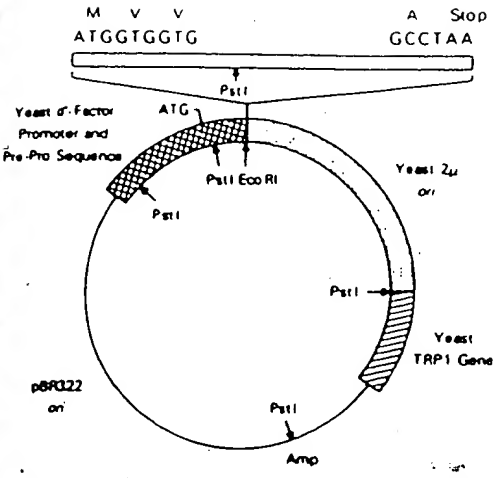
FIG. 1. Mutations in the carboxy terminus of human TGF-α. The amino acids conserved in all the family of EGF-like growth factors (human and murine EGFs and TGFs, as well as the gene products of the vaccinia virus [vaccinia growth factor], the Shope fibroma virus [Shope fibroma growth factor], and the myxoma virus [myxoma growth factor]) are enclosed in bold circles. The mutations of amino acids at positions 47 and 48 are indicated. Symbols: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

Asp-47 has been mutated to Glu, Asn, Ser, and Ala. Glu was chosen because it has the same charge as and a larger size than Asp; Asn has a similar side-chain structure, but is uncharged; Ser is smaller but still polar; Ala is smaller and nonpolar.

Leu 48 has been mutated to Ile and Met, which are both large, nonpolar residues like Leu, and to Ala, which is nonpolar but smaller. We introduced the chosen mutations by site-directed mutagenesis of the cloned human TGF-α gene, using synthetic oligonucleotides.

Construction of the yeast α mating pheromone–human TGF-α plasmid. The TGF-α expression vector pyTE1 (Fig. 2) was constructed by using plasmid YEp70αT (15) which contains the 2μm origin of replication and yeast *TRP1* gene for its replication and selective maintenance, respectively. YEp70αT also contains the yeast α-factor promoter, the α-factor prepro sequence coding for 89 amino acids, and the sequence for 3 amino acids resulting from the introduction of *Xba*I and *Eco*RI sites. The human mature TGF-α sequence (12) is contained in a 170-base-pair *Eco*RI fragment which includes an ATG (Met) codon preceding the sequence of TGF-α and a TAA (stop) codon followed by 8 nucleotides. This TGF-α sequence was inserted in the unique *Eco*RI site of YEp70αT. Clones with the proper orientation were selected, and DNA was isolated for yeast transformation.

Measurement of TGF-α secreted by *S. cerevisiae*. The amount of total proteins secreted into the yeast culture was 10 ± 1 μg/ml for wild-type as well as mutant TGF-α as determined by the method of Bradford (3). Before further purification was attempted, we wanted to determine whether the mutated TGF-α proteins were being secreted by the yeast. The low pH of the yeast medium, as well as the acidic proteins secreted in the yeast culture, precluded biological assay of secreted mutants. Therefore, immunological meth-



G. 2. Structure of the *S. cerevisiae* 8.2-kilobase shuttle vector 1. The secretion of the TGF-α gene is under the transcriptional l of the yeast α-factor promoter and prepro sequence ( ). east 2μm origin of replication ( ) and the selective yeast gene ( ) are indicated. The TGF-α gene, preceded by an ion (ATG) codon and followed by a stop (TAA) codon, is d in the *Eco*RI site. Details are given in Materials and ods and in Results.

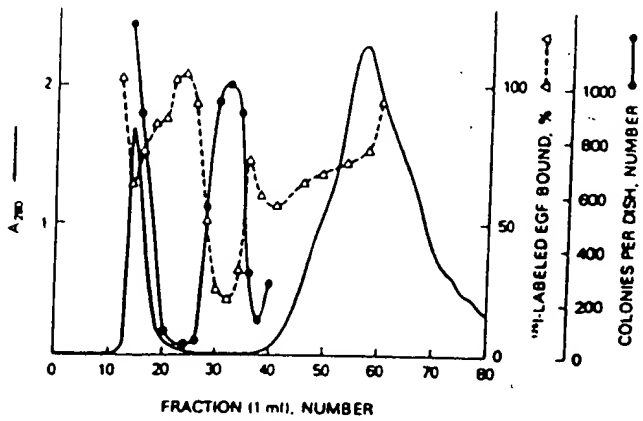


FIG. 3. Purification of yeast-secreted wild-type TGF-α. The purification procedure is described in Materials and Methods and in Results. Aliquots of every other fraction of the Bio-Gel P30 column were tested for their abilities to compete with ^{125}I-EGF for binding to the EGF receptor (Δ) and to induce colony formation (>62 μm) on NRK cells in soft agar in the presence of TGF-β (1 ng/ml) (●). The A_{280} profile of the proteins was determined (——).

ere used. Wild-type and mutant TGF-α's were se- at a level of 100 to 200 ng/ml and 10 to 500 ng/ml. ctively (as determined by radioimmunoassay with poly- antibody 34D). We thus estimate that the percentage F-α secreted in the yeast culture is at least 1% of the rotein secreted. We cannot yet assess whether the ions in the levels of secretion of different mutant α proteins are real or whether one single-amino-acid tution drastically affects the recognition by the anti- The latter hypothesis is the more likely, since the use ther polyclonal antibody (Biotope) under denaturing ions enabled us to detect certain TGF-α mutants (such a 47]-TGF-α, in which the amino acid in position 47 of TGF-α is mutated to an alanine) that were poorly ed by 34D, under nondenaturing as well as denaturing ions. After the amount of TGF-α mutant proteins was ated, the medium was extensively dialyzed against 1 M acid and lyophilized as described in Materials and ods.

tial purification of yeast-secreted TGF-α. Although the shuttle vector was constructed in such a way as to e TGF-α with 8 amino acids fused to the N terminus, s often observed that a significant fraction of the ed TGF-α was in a higher-molecular-weight fragment sponding to the size expected from an uncleaved fun- sed) 92-amino-acid fusion protein. Since a Met had introduced at the N terminus of TGF-α and since contains no Met in its sequence, CNBr treatment be used to cleave either of these 8- or 92-amino-acid minal peptides and release the complete 50-amino-acid . Indeed, CNBr treatment of yeast-secreted proteins d in the conversion of high-molecular-weight TGF-α e 6,000-molecular-weight species, as revealed by n immunoblot (data not shown).

r-cleaved samples (see Materials and Methods) were on a Bio-Gel P30 column. Figure 3 shows the elution of the proteins, as well as the results of a radiorecep- y and a soft-agar assay performed on aliquots of the fractions. The A_{280} profile shows two major peaks of eluted proteins, one corresponding to the void volume and the other one to proteins of molecular weight <3,000. Aliquots of the column fractions were tested for their ability to compete with ^{125}I-EGF for binding to the receptor. The fractions that were the most active in this assay were located between the two major protein peaks, in an area where relatively few proteins eluted. Although some activity was found in the first protein peak (void volume), this was considerably reduced on treatment with stronger CNBr (data not shown).

Aliquots of each fraction were also tested for their ability to induce anchorage-independent growth of NRK cells in soft agar in the presence of TGF-β (1 ng/ml). The receptor binding and colony-forming activity superimposed almost exactly (Fig. 3). Analysis by polyacrylamide gel electrophoresis with silver staining, as well as by Western blot, of the column fractions shows that our purification procedure (CNBr cleavage followed by P30 sizing column) eliminates high-molecular-weight proteins (data not shown). Since pure TGF-α migrates in a broad band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (32), this technique cannot be used for proper assessment of the degree of separation of TGF-α from low-molecular-weight contaminating proteins. Nevertheless, within our detection levels the amounts of TGF-α present in the column fractions (detected by radioimmunoassay using the antibody from Biotope) correlated with the amounts observed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

Comparison of binding and colony-forming activity of TGF-α partially purified from yeast media. It was important to show that wild-type TGF-α secreted from *S. cerevisiae* had the expected biological properties and that its activity in soft-agar and radioreceptor assays was equivalent. For these assays, the amount of EGF-competing activity present in the most active fraction of the P30 column of wild-type TGF-α was measured in terms of EGF equivalents. The dilution curve had a slope that was parallel to that of the EGF standard. This value was also used to measure the colony-forming activity of the partially purified wild-type TGF-α (with EGF as a standard in the assay). The colony-forming activity of the partially purified wild-type TGF-α corre-

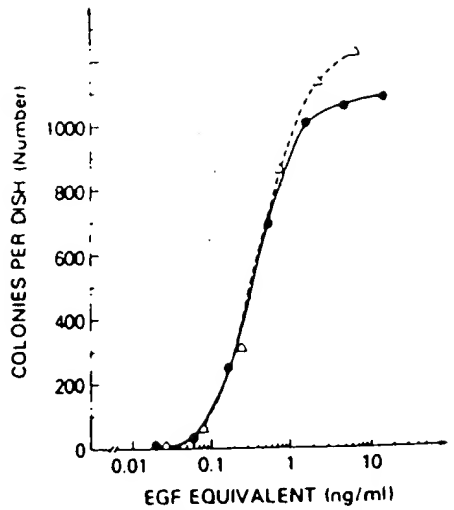


FIG. 4. Correlation between the activities in the binding and colony-forming assay for the partially purified wild-type TGF-α secreted by *S. cerevisiae*. The activity in the radioreceptor assay of the peak fraction from the P30 column was determined in EGF equivalent concentration. The value obtained was used for the soft-agar assay. Colonies of >62 μm (△) and the EGF standard (●) are shown.

sponded exactly to that of EGF (Fig. 4). Thus, we have partially purified a wild-type 50-amino-acid TGF-α showing the expected binding and colony-forming activities, which provides a reference substance for mutant TGF-αs that might show a dissociation of binding and colony-forming abilities.

Biological and biochemical activities of the partially purified TGF-α mutant proteins. Mutated TGF-αs were expressed by using the yeast system and partially purified on Bio-Gel P30 columns as described in Materials and Methods. Mutant TGF-αs were usually obtained from two different clones of yeast transformants. The CNBr-cleaved samples were purified through different Bio-Gel P30 columns for each mutant protein to avoid any possible contamination from one peptide to another. The purification profiles observed with the mutant TGF-αs were similar to those obtained for the wild-type TGF-α. Aliquots of the P30 column fractions were tested in radioreceptor and soft-agar assays. For all mutant proteins, the highest activity in both assays was always found in the same fraction of the Bio-Gel P30 column effluent (peak fraction). Extensive purification of a series of mutant proteins for screening purposes is not practical. Therefore, we needed a quantitation system that would allow us to compare mutant proteins with each other. Thus, the amount of TGF-α present in the peak fraction was estimated by radioimmunoassay with an antiserum to native TGF-α (obtained from W. Hargreaves), under denaturing conditions, as described in Materials and Methods. All values given in Table 1 were obtained from the peak fraction.

The controls done with the wild-type TGF-α showed (Fig. 4; Table 1) that binding and transforming activity were equivalent. The yeast vector without a TGF-α insert did not secrete any EGF-like proteins, as determined by both radioreceptor and soft-agar assay.

Two types of results were obtained upon assay of mutant proteins having different amino acid substitutions at Asp-47. In both [Ala-47]–TGF-α and [Asn-47]–TGF-α, binding ability was retained. Soft-agar and radioreceptor activities correlated for [Asn-47]–TGF-α; there was a lower value for

TABLE 1. Biological and biochemical activities of mutant TGF-α proteins secreted by *S. cerevisiae* and partially purified

Insert in the yeast expression vector	EGF equivalence (ng/ml) in:		Amt of TGF-α (ng/ml) in radioimmunoassay
	Radioreceptor assay	Soft-agar assay	
Wild-type TGF-α	700	700	2,000
	400	300	ND[a]
None	0	0	0
[Ala-47]–TGF-α	100	44	220
	66	48	ND
[Asn-47]–TGF-α	80	72	180
	75	72	525
[Glu-47]–TGF-α	3	3	42
[Ser-47]–TGF-α	10	4	60
[Ala-48]–TGF-α	0	0	16
	0	0	220
[Ile-48]–TGF-α	4	12	470
	2	7	490
[Met-48]–TGF-α	2	8	453
	0.5	2	420

[a] ND, Not determined.

colony-forming activity than for EGF-binding competition for [Ala-47]–TGF-α. [Ser-47]–TGF-α and [Glu-47]–TGF-α appeared to have lower activities in both assays than either wild-type TGF-α or [Ala-47]–TGF-α and [Asn-47]–TGF-α. These results indicate that neither the carboxyl charge nor the polarity of Asp-47 is essential for biological activity.

The effects of mutation of Leu-48, one of the 11 amino acids perfectly conserved among all the EGFs, TGF-αs, and viral EGF-like proteins, are dramatic. [Ala-48]–TGF-α totally lacked binding and colony-forming activity. [Ile-48]–TGF-α and [Met-48]–TGF-α had very little biological activity compared with wild-type TGF-α. Another substitution, [Met-48]–TGF-α, resulted in a truncated mutant lacking the last 2 amino acids and having a substitution of Leu to homoserine at position 48 following treatment with CNBr. Alternatively, if [Met-48]–TGF-α was not treated with CNBr, fusion proteins of TGF-α (mutated to Met in position 48) with 8 or 92 amino acids attached at the N terminus were obtained. Very low activities in binding and soft-agar assays were found for these mutants, whether or not they were cleaved with CNBr. Experiments on EGF and TGF-α have shown that an N-terminal extension does not markedly modify EGF-binding activity (12, 26). Therefore, the loss of activity obtained with [Met-48]–TGF-α that has not been CNBr treated was probably due to the mutation itself and not to the N-terminally extended fusion protein. We do not know whether the loss of activity observed with the TGF-α shortened to 48 amino acids and having a substitution of Leu-48 to homoserine is due only to the mutation or also to the lack of the last 2 amino acids.

The data obtained by radioimmunoassay on the partially purified wild-type and mutant TGF-α show that the amount of TGF-α detected was always higher than the amount determined by measurement of biological activity. This may be due to the presence in the fraction of a certain percentage of incorrectly folded TGF-α that might be recognized in a

radioimmunoassay under denaturing conditions but would not be biologically active. None of the mutant proteins seemed to be present in amounts equivalent to those observed for wild-type TGF-α in the partially purified fractions (whether radioimmunoassay, radioreceptor, or soft-agar assay was used for quantitation). It is not clear whether consistently less TGF-α was produced by the mutant constructs than by the wild type or whether the secreted mutant proteins were simply less well recognized by the antibody. Because of these uncertainties, the biological activities of the different mutant proteins cannot be accurately related to a known amount of mutant TGF-α protein. Even though radioimmunoassay should be used with caution for a quantitative evaluation of mutant TGF-α proteins, a positive reaction demonstrates that immunoreactive TGF-α was present in the P30 peak fraction for each mutant. Therefore, the fact that one of the mutant proteins ([Ala-48]-TGF-α) is biologically inactive can be attributed to the mutation itself, and not to the lack of production of the mutant protein by the yeast or its loss through purification. However, if the mutant proteins are in fact as immunoreactive as the wild type, then [Ala-47]-TGF-α and [Asn-47]-TGF-α are as active as wild-type TGF-α and [Glu-47]-TGF-α and [Ser-47]-TGF-α are less active; in contrast, [Ile-48]-TGF-α and [Met-48]-TGF-α are almost inactive. The differences between mutation of Asp-47 and Leu-48 would then be even more striking.

DISCUSSION

TGF-α shows sequence homologies with EGF, and both growth factors share the same cellular receptors (20). Even though EGF was discovered 25 years ago (7) and its properties have been extensively studied over the years (5), the binding site of EGF to its receptor has still not been determined, and the relationship between structure and function of EGF/TGF-α is still to be discovered. Particularly, we do not know whether binding to the receptor and cellular transduction occur through one or more domains of the molecule or through which amino acids. We approached the question by performing site-directed mutagenesis of TGF-α and focused our attention on two adjacent amino acids, Asp-47 and Leu-48, located in the carboxy terminus and highly conserved in the EGF-like family of peptides. Unexpectedly, these two amino acids showed very different sensitivities to mutation and particularly to a substitution to Ala: [Ala-47]-TGF-α retained binding and colony-forming activities, whereas [Ala-48]-TGF-α completely lost both activities. These data show that Asp-47 and Leu-48 play very different roles in defining the structure and/or the activity of TGF-α. The other mutations performed on Asp-47 were substitutions to Asn, Ser, and Glu. [Asn-47]-TGF-α, like [Ala-47]-TGF-α, was active in binding and induction of colony formation, but [Ser-47]-TGF-α and [Glu-47]-TGF-α showed weaker growth factor activities. These results indicate that neither the carboxyl charge nor the polarity of Asp-47 is essential for biological activity. Interestingly, two of the EGF-like viral proteins, myxoma growth factor and Shope fibroma growth factor (6, 30), have Asn instead of Asp in position 47; we have shown that [Asn-47]-TGF-α retains biological activity.

Substitution of Leu-48 to Met and Ile led to mutant proteins with very low activities, whereas substitution to Ala led to complete loss of activity. We did not expect that a substitution of Leu to Ile (which have similar sizes and polarity) would cause such a strong effect. Thus, Leu-48, which is conserved perfectly among all the EGF-like peptides, seems to be essential, through its exact geometry, for the biological activity of TGF-α.

The mutant proteins tested so far, when active, showed parallel behaviors in binding and colony formation. Some mutant proteins lost all activities, and we assume that the binding capacity has been lost. We have not been able to dissociate the binding and colony-forming abilities by using any of the present series of mutant proteins, and it is necessary to screen more of them in search of an antagonist of TGF-α.

Results relating to the biological activity of EGF show that derivatives of mouse EGF and human EGF (EGF 1-47) lacking the carboxy-terminal 6 amino acids as a result of enzymatic digestion are less potent than the intact molecule in mitogenic stimulation of fibroblasts, but retain full biological activity in in vivo assays (inhibition of gastric acid secretion) (16). On the other hand, naturally occurring truncated forms of rat EGF, which lack the carboxy-terminal 5 amino acids (rEGF 2-48) are as potent as mouse EGF (mEGF 1-53) in receptor-binding and mitogenic assays (27). We do not know whether the discrepancies observed are due to the origin of the molecule (artificial or natural) or to the type of bioassay used. In any event, all of these EGF-related molecules, which are shorter than mouse or human EGF, still retain Leu-47. We have shown that in TGF-α, the corresponding residue, Leu-48, is critical for the biological activity.

Recent data on the three-dimensional structure of mouse EGF obtained by nuclear magnetic resonance show that even though Asp-46 and Leu-47 (Asp-47 and Leu-48 in TGF-α) are both solvent accessible (8, 22, 22a), their side chains point in opposite directions in the beta-sheet structure. Therefore, the role of these adjacent amino acids in the structure and, consequently, the function of EGF might be very different. Our data show that the amino acids Asp-47 and Leu-48 of TGF-α are not equally important for the biological activity of TGF-α, despite their conservation among the EGF-like peptides. From the dramatic loss in biological activity which is characteristic of mutation of Leu-48, we also suggest that this residue is involved in binding to the cellular receptors either by direct interaction with the receptor or by providing the proper conformation to the molecule.

ACKNOWLEDGMENTS

We thank Rik Derynck (Genentech) for providing the TGF-α gene, inserted in M13, and for his assistance throughout this project. We are indebted to Arjun Singh (Genentech) for helping us with the yeast transformation and expression. We are grateful to Tim Bringman (Genentech) and William Hargreaves (Biotope) for their generous gifts of TGF-α antibodies. We thank Linda Durham for technical assistance, Irene Dalton for manuscript preparation, and our colleagues for helpful comments and moral support.

LITERATURE CITED

1. Anzano, M. A., A. B. Roberts, J. M. Smith, M. B. Sporn, and J. E. De Larco. 1983. Sarcoma growth factor from conditioned medium of virally transformed cells is composed of both type α and type β transforming growth factors. Proc. Natl. Acad. Sci. USA 80:6264–6268.
2. Blomquist, M. C., L. T. Hunt, and W. C. Barker. 1984. Vaccinia virus 19-kilodalton protein: relationship to several mammalian proteins, including two growth factors. Proc. Natl. Acad. Sci. USA 81:7363–7367.
3. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.

4. **Bringman, T. S., P. B. Lindquist, and R. Derynck.** 1987. Different transforming growth factor-α species are derived from a glycosylated and palmitoylated transmembrane precursor. Cell 48:429–440.
5. **Carpenter, G., and S. Cohen.** 1979. Epidermal growth factor. Annu. Rev. Biochem. 48:193–216.
6. **Chang, W., C. Upton, S. Hu, A. F. Purchio, and G. McFadden.** 1987. The genome of Shope fibroma virus, a tumorigenic poxvirus, contains a growth factor gene with sequence similarity to those encoding epidermal growth factor and transforming growth factor α. Mol. Cell. Biol. 7:535–540.
7. **Cohen, S.** 1962. Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. J. Biol. Chem. 237:1555–1562.
8. **Cooke, R. M., A. J. Wilkinson, M. Baron, A. Pastore, M. J. Tappin, I. D. Campbell, H. Gregory, and B. Sheard.** 1987. The solution structure of human epidermal growth factor. Nature (London) 327:339–341.
9. **De Larco, J. E., and G. J. Todaro.** 1978. Growth factors from murine sarcoma virus-transformed cells. Proc. Natl. Acad. Sci. USA 75:4001–4005.
10. **Derynck, R., D. V. Goeddel, A. Ullrich, J. C. Gutterman, R. D. Williams, T. S. Bringman, and W. H. Berger.** 1987. Synthesis of messenger-RNAs for transforming growth factors α and β and the epidermal growth factor receptor by human tumors. Cancer Res. 47:707–712.
11. **Derynck, R., A. B. Roberts, D. H. Eaton, M. E. Winkler, and D. V. Goeddel.** 1985. Human transforming growth factor-α: precursor sequence, gene structure, and heterologous expression. Cancer Cells 3:79–86.
12. **Derynck, R., A. B. Roberts, M. E. Winkler, E. Y. Chen, and D. V. Goeddel.** 1984. Human transforming growth factor-α: Precursor structure and expression in E. coli. Cell 38:287–297.
13. **Gregory, H.** 1975. Isolation and structure of urogastrone and its relationship to epidermal growth factor. Nature (London) 257:325–327.
14. **Hinnen, A., J. B. Hicks, and G. R. Fink.** 1978. Transformation of yeast. Proc. Natl. Acad. Sci. USA 75:1929–1933.
15. **Hitzeman, R. A., C. N. Chang, M. Matteucci, L. J. Perry, W. J. Kohr, J. J. Wulf, J. R. Swartz, C. Y. Chen, and A. Singh.** 1986. Construction of expression vectors for secretion of human interferons by yeast. Methods Enzymol. 119:424–433.
16. **Hollenberg, M. D., and H. Gregory.** 1980. Epidermal growth factor—urogastrone: biological activity and receptor binding of derivatives. Mol. Pharmacol. 17:314–320.
17. **Jones, E.** 1976. Proteinase mutants of *Saccharomyces cerevisiae*. Genetics 85:23–30.
18. **Lee, D. C., R. Rochford, G. J. Todaro, and L. P. Villarreal.** 1985. Developmental expression of rat transforming growth factor α mRNA. Mol. Cell. Biol. 5:3644–3646.
19. **Marquardt, H., M. W. Hunkapiller, L. E. Hood, and G. J. Todaro.** 1984. Rat transforming growth factor type 1: structure and relation to epidermal growth factor. Science 223:1079–1082.
20. **Massague, J.** 1983. Epidermal growth factor-like transforming growth factor. II. Interaction with epidermal growth factor receptors in human placenta membranes and A431 cells. J. Biol. Chem. 258:13614–13620.
21. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. 101:20–78.
22. **Montelione, G. T., K. Wüthrich, E. C. Nice, A. W. Burgess, and H. A. Scheraga.** 1986. Identification of two anti-parallel beta-sheet conformations in the solution structure of murine epidermal growth factor by proton magnetic resonance. Proc. Natl. Acad. Sci. USA 83:8594–8598.
22a. **Montelione, G. J., K. Wuthrich, E. C. Nice, A. W. Burgess, and H. A. Scheraga.** 1987. Solution structure of murine epidermal growth factor: determination of the polypeptide backbone chain-fold by nuclear magnetic resonance and distance geometry. Proc. Natl. Acad. Sci. USA 84:5226–5230.
23. **Samsoondar, J., M. S. Kobrin, and J. E. Kudlow.** 1986. Transforming growth factor secreted by untransformed bovine anterior pituitary cells in culture. J. Biol. Chem. 261:14408–14413.
24. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463–5467.
25. **Savage, C. R., Jr., J. H. Hash, and S. Cohen.** 1973. Epidermal growth factor: location of disulfide bonds. J. Biol. Chem. 248:7669–7672.
26. **Shechter, Y., J. Schlessinger, S. Jacobs, K. J. Chang, and P. Cuatrecasas.** 1978. Fluorescent labeling of hormone receptors in viable cells: preparation and properties of highly fluorescent derivatives of epidermal growth factor and insulin. Proc. Natl. Acad. Sci. USA 75:2135–2139.
27. **Simpson, R. J., J. A. Smith, R. L. Moritz, M. J. O'Hare, P. S. Rudland, J. R. Morrison, C. J. Lloyd, B. Grego, A. W. Burgess, and E. C. Nice.** 1985. Rat epidermal growth factor: complete amino acid sequence. Eur. J. Biochem. 153:629–637.
28. **Singh, A., J. M. Lugovoy, W. J. Kohr, and L. J. Perry.** 1984. Synthesis, secretion and processing of α-factor-interferon fusion proteins in yeast. Nucleic Acids Res. 12:8927–8938.
29. **Todaro, G. J., C. Fryling, and J. E. DeLarco.** 1980. Transforming growth factors produced by certain human tumors: polypeptides that interact with epidermal growth factor receptors. Proc. Natl. Acad. Sci. USA 77:5258–5262.
30. **Upton, C., J. L. Macen, and G. McFadden.** 1987. Mapping and sequencing of a gene from myxoma virus that is related to those encoding epidermal growth factor and transforming growth factor α. J. Virol. 61:1271–1275.
31. **Watanabe, S., E. Lazar, and M. B. Sporn.** 1987. Transformation of normal rat kidney (NRK) cells by an infectious retrovirus carrying a synthetic rat type α transforming growth factor gene. Proc. Natl. Acad. Sci. USA 84:1258–1262.
32. **Winkler, M. E., T. Bringman, and B. J. Marks.** 1986. The purification of fully active recombinant transforming growth factor-α produced in *Escherichia coli*. J. Biol. Chem. 261:13838–13843.
33. **Zoller, M. J., and M. Smith.** 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. Methods Enzymol. 101:468–500.

Molecular Microbiology (1991) 5, 1765–1767 ADONIS 0950382X91001957

Analysis of structure and function of the B subunit of cholera toxin by the use of site-directed mutagenesis

M. G. Jobling and R. K. Holmes*
Department of Microbiology, F. Edward Herbert School of Medicine, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, Maryland 20814-4799, USA.

Summary

Oligonucleotide-directed mutagenesis of *ctxB* was used to produce mutants of cholera toxin B subunit (CT-B) altered at residues Cys-9, Gly-33, Lys-34, Arg-35, Cys-86 and Trp-88. Mutants were identified phenotypically by radial passive immune haemolysis assays and genotypically by colony hybridization with specific oligonucleotide probes. Mutant CT-B polypeptides were characterized for immunoreactivity, binding to ganglioside GM1, ability to associate with the A subunit, ability to form holotoxin, and biological activity. Amino acid substitutions that caused decreased binding of mutant CT-B to ganglioside GM1 and abolished toxicity included negatively charged or large hydrophobic residues for Gly-33 and negatively or positively charged residues for Trp-88. Substitution of lysine or arginine for Gly-33 did not affect immunoreactivity or GM1-binding activity of CT-B but abolished or reduced toxicity of the mutant holotoxins, respectively. Substitutions of Glu or Asp for Arg-35 interfered with formation of holotoxin, but none of the observed substitutions for Lys-34 or Arg-35 affected binding of CT-B to GM1. The Cys-9, Cys-86 and Trp-88 residues were important for establishing or maintaining the native conformation of CT-B or protecting the CT-B polypeptide from rapid degradation *in vivo*.

Introduction

The symptoms of cholera, an acute diarrhoeal disease of humans caused by *Vibrio cholerae*, are due mainly to the effects of cholera toxin (CT) on the small intestine. CT and the heat-labile enterotoxins (LT) of *Escherichia coli* are structurally and functionally related and constitute the *V. cholerae/E. coli* enterotoxin family (reviewed by Finkelstein *et al.*, 1987). Type I and type II enterotoxins belong to distinct serogroups, and minor antigenic variants exist within each serogroup (Holmes *et al.*, 1986; Guth *et al.*, 1986; Pickett *et al.*, 1987; 1989). The sequences of the structural genes for representative type I and type II enterotoxins have been determined (reviewed by Betley *et al.*, 1986; Pickett *et al.*, 1987; 1989). CT and LT are multisubunit toxins of the A–B type (Olsnes *et al.*, 1990). CT is an 85 kDa complex containing a 27 kDa A polypeptide and five 11.6 kDa B polypeptides. The mature CT-B monomer consists of 103 amino acid residues (Lai, 1977), with a single intrachain disulphide link between Cys-9 and Cys-86. There are five ganglioside GM1 (GM1)-binding sites per molecule of CT (Fishman *et al.*, 1978). Interaction of the B pentamer of CT with GM1 on the plasma membrane of target cells initiates delivery of the A polypeptide to the cytosol. The A subunit is proteolytically cleaved to produce two polypeptides (A1 and A2) of 21.8 and 5.4 kDa, linked by a disulphide bond. The A1 polypeptide is the enzymatically active component of the toxin, catalysing the transfer of the ADP-ribose moiety from NAD to the α subunit of the Gs regulatory protein of adenylate cyclase. This activates adenylate cyclase, increases production of cAMP, and causes secretion of fluid and electrolytes by the small intestine (Field *et al.*, 1989).

Attempts to characterize the ganglioside-binding sites of CT and LT have concentrated on analysis of chemically modified toxins and on the isolation of mutants. However, the precise nature of the ganglioside-binding sites on CT-B and LT-B remains to be elucidated.

Several lines of evidence show that Trp-88 is closely associated with the GM1-binding site. Binding of GM1 to CT causes a blue shift of approximately 12 nm in the fluorescence emission maximum of the single tryptophan residue (Mullin *et al.*, 1976), and modification of tryptophan with nitrophenyl derivatives (de Wolf *et al.*, 1981) or formic acid (Ludwig *et al.*, 1985) destroys the ability of CT to bind to GM1. One or more of the nine lysine residues in CT-B appear(s) to be involved in the GM1 binding site (Ludwig *et al.*, 1985). Modification of CT-B with cyclohexanedione led Duffy and Lai (1979) to conclude that Arg-35 is involved in receptor binding, but Ludwig *et al.* (1985) could not attribute the effects of arginine modification to a specific residue from the three arginines in CT-B. The disulphide bond between Cys-9 and Cys-86 is important for the structural integrity of CT-B, and modification of

Received 8 February, 1991. *For correspondence. Tel. (301) 295 3400.

these cysteines destroys both GM1-binding activity and antigenicity of CT-B (Ludwig *et al.*, 1985).

Several factors can complicate the interpretation of data from chemical modification studies. Either the conditions necessary to perform the modification or the modified amino acids themselves could alter CT-B in such a way that refolding into a native state is no longer possible. Inability to modify a particular residue among several identical residues may prevent identification of the importance of an individual residue. Incomplete modification could lead to failure to identify specific residues as essential for biological activity. Mutational analysis avoids some of these problems and provides data that complement and extend the results of chemical modification. The study of mutants with defined substitutions of naturally occurring amino acids for specific wild-type residues facilitates interpretation of the biochemical basis for mutant phenotypes.

Several variants of CT-B have been identified that differ in two or three residues (reviewed in Finkelstein *et al.*, 1987). LTp-I and LTh-I differ in terms of their B subunits at four or five positions (Tsuji *et al.*, 1987; Leong *et al.*, 1986), and differ from CT-B at approximately 25% of the positions. All of these enterotoxins bind to GM1, but the type I LTs also bind to several glycoprotein receptors to which CT does not bind (Griffiths *et al.*, 1986). Mutant B subunits of LTp-I have been isolated that are defective in forming pentamers (Ala-64 to Val-64, Iida *et al.*, 1989; carboxyl-terminal deletions, Sandkvist *et al.*, 1990) or that are defective in binding to GM1 (glycine to aspartate-33; Tsuji *et al.*, 1985). The Asp-33 mutant of LT-B is unimpaired in its ability to form holotoxin but is non-toxic. The Gly-33 and the Ala-64 residues are both conserved in CT-B.

The role of any specific amino acid residue in the ganglioside-binding activity of CT-B can be determined, in principal, by observing which substitutions at that position are tolerated in mutant proteins without loss of function. In the present study we used site-directed mutagenesis to create substitution mutants involving Cys-9, Cys-86, Gly-33, Lys-34, Arg-35 and Trp-88. Preliminary reports involving some of these mutants were presented at the 24th and 26th Joint Conferences on Cholera and Related Diarrheal Diseases, US–Japan Co-operative Medical Science Programme (Jobling *et al.*, 1991a,b).

Results

The mutagenic oligonucleotides used in this study are listed in Table 1. Plasmids pMGJ8 and pMGJ19 were constructed as described in the *Experimental procedures*. Each plasmid contains the cloned cholera toxin (*ctx*) operon with an in-frame deletion that removes the majority of the A1 coding region but does not affect the B gene (*ctxA*$^-$, *ctxB*$^+$). Transcription of the operon from the native *V. cholerae* promoter occurs at a low level in *E. coli*. To increase the expression of wild-type or mutant *ctxB* alleles in *E. coli*, *toxR* was provided on plasmid pVM25 (Miller and Mekalanos, 1984). Mutant plasmids were numbered sequentially, beginning with pMGJ801 for plasmids

Table 1. Mutagenic oligonucleotides used to create missense mutations affecting specific residues in the B polypeptide of cholera toxin.

Oligonucleotide[a]	Sequence (5′ to 3′)[b]	Template	Strand[c] rescued
Cys-9-TCA	T T C T G C A C T C A A A T C A G T (A)	pMGJ8	−
Cys-86-AGT	G A A A A G T T A A G T G T A T G G (T)	pMGJ19	+
Gly-33-NNN	T C T C T A G C T N N N A A A A G A G A G (G G A)	pMGJ19	+
Lys-34-NNC	C T A G C T G G A N N C A G A G A G A T G (A A A)	pMGJ11	+
Arg-35-NNN	G C T G G A A A A N N N G A G A T G G C T (A G A)	pMGJ19	+
Trp-88-NNN	T T A T G T G T A N N N A A T A A T A A A A C G C C (T G G)	pMGJ19	+

a. Each oligonucleotide is named by the wild-type amino acid in mature CT-B that is affected, followed by the unique or degenerate codon used to mutate that residue in the specified template.
b. The mutant sequence is underlined and the wild-type sequence is shown beneath in parentheses. N represents an equal mixture of A, G, C or T.
c. + or − denotes the sense or antisense strand, respectively, of the CT-B gene rescued and used as the template for mutagenesis with the corresponding oligonucleotide.

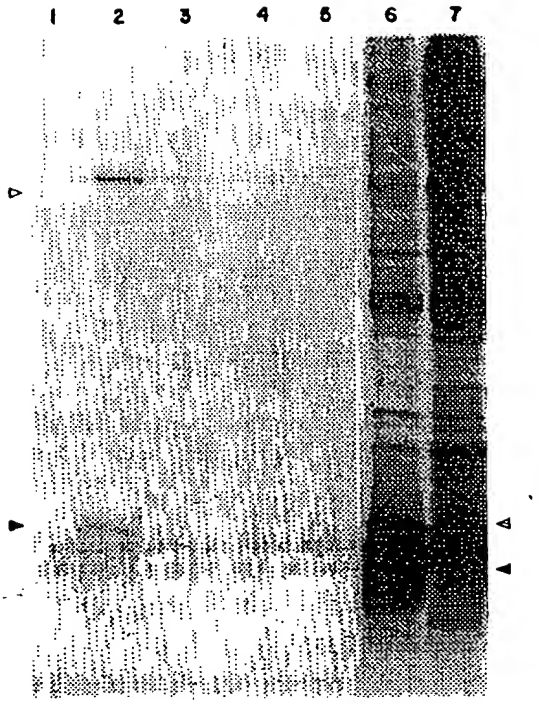


Fig. 1. [^{35}S]-methionine-labelled proteins expressed from the T7 promoter by induction of T7 RNA polymerase in *E. coli* strains bearing plasmids that code for cysteine mutants of CT-B. Lanes 1–5, periplasmic extracts; lanes 6 and 7, cell pellets. Lane 1, vector control, pKS$^-$; lanes 2 and 6, wild-type, pMGJ19; lane 3, Ser-9, pMGJ801; lane 4, Ser-86, pMGJ1901; lanes 5 and 7, Ser-9, −86, pMGJ20. Arrowheads mark approximate positions of pentameric CT-B (open), monomeric CT-B (filled), and precursor CT-B (hatched).

derived from pMGJ8, with pMGJ1901 for plasmids derived from pMGJ19, and with pMGJ1101 for derivatives of pMGJ11.

Cys-9 and Cys-86 are essential for production of mature periplasmic CT-B in E. coli

Oligonucleotide-directed site-specific mutagenesis was used to mutate each cysteine to serine. Cys-9 was mutated to Ser in pMGJ801 (TGT to AGT, using oligonucleotide Cys-9-TCA), and Cys-86 was mutated to Ser in pMGJ1901 (TGT to AGT) using the oligonucleotide Cys-86-AGT (Table 1). The Ser-9, Ser-86 double mutant, pMGJ20, was constructed by replacing an *Acc*I fragment of pMGJ1901 with the corresponding *Acc*I fragment from pMGJ801. *E. coli* DH5αF′(pVM25) carrying each of these plasmids gave no halo when tested with the radial passive immune haemolysis assay (RPIHA) for CT-B. No immunoreactive CT-B protein was detected in crude extracts of the mutant strains by the more-sensitive double antibody sandwich solid-phase radioimmunoassay (S-SPRIA). Analysis of *in vivo* labelled proteins by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS–PAGE) showed that mature CT-B polypeptide (monomer or pentamer) was undetectable in periplasmic extracts of any of the cysteine mutants under conditions in which wild-type protein was detected (Fig. 1). A band corresponding to unprocessed CT-B polypeptide was detected in the pellet of polymyxin-B-extracted cells of the Ser-9, Ser-86 double mutant. The Ser-9 and Ser-86 single mutants were not tested for intracellular CT-B.

Effects of amino acid substitutions for glycine-33

Using a 64-fold degenerate oligonucleotide (Gly-33-NNN, Table 1) we generated a library of mutants of pMGJ19 potentially containing all possible substitutions for Gly-33. One hundred and ninety two individual transformants were screened for loss of receptor-binding activity by RPIHA and for base substitutions at the Gly-33 codon in colony hybridizations using an end-labelled wild-type oligonucleotide probe. The phenotypes of representative mutants in RPIH assays are shown in Fig. 2. Among 25 isolates with mutant nucleotide sequences, only three were positive by RPIHA. These three mutants all contained alternative glycine codons, strongly suggesting that only CT-B containing glycine at position 33 can produce a positive signal in RPIHA. Among the 22 RPIHA-negative mutants, 20 produced mutant CT-Bs with amino acid substitutions, and two were chain-terminating mutants. The set of 20 substitutions represented 11 of the possible 19 amino acid substitutions (Table 2). One mutant representing each substitution was selected for further study. All produced high levels of immunoreactive protein as measured by S-SPRIA (Fig. 3A). In contrast, the GM1-binding activity, as measured by GM1-SPRIA, varied greatly among the individual mutants (Fig. 3B).

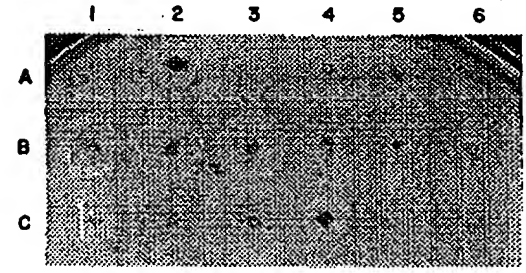


Fig. 2. RPIHA phenotype of selected mutant B subunit producing clones. *E. coli* strains carrying pVM25 and plasmids encoding various CT-B mutants were inoculated into an SRBC overlay on an LB (Ap, Cm) plate and grown overnight at 37°C. A second overlay containing Guinea-Pig Complement (BRL) and goat anti-CT serum was poured and incubated for at least 1 h at 37°C to produce visible haloes. Clones tested were: wild type (A2), Leu-33 (A3), Arg-33 (A4), Glu-33 (A5), Met-35 (B1), Tyr-35 (B2), Asn-35 (B3), Asn-88 (B4), Lys-88 (B5), Glu-88 (B6), Cys-35 (C1), Asp-35 (C2), Glu-35 (C3), wild type (C4), pKS$^-$ (C5) and Glu-88 (C6).

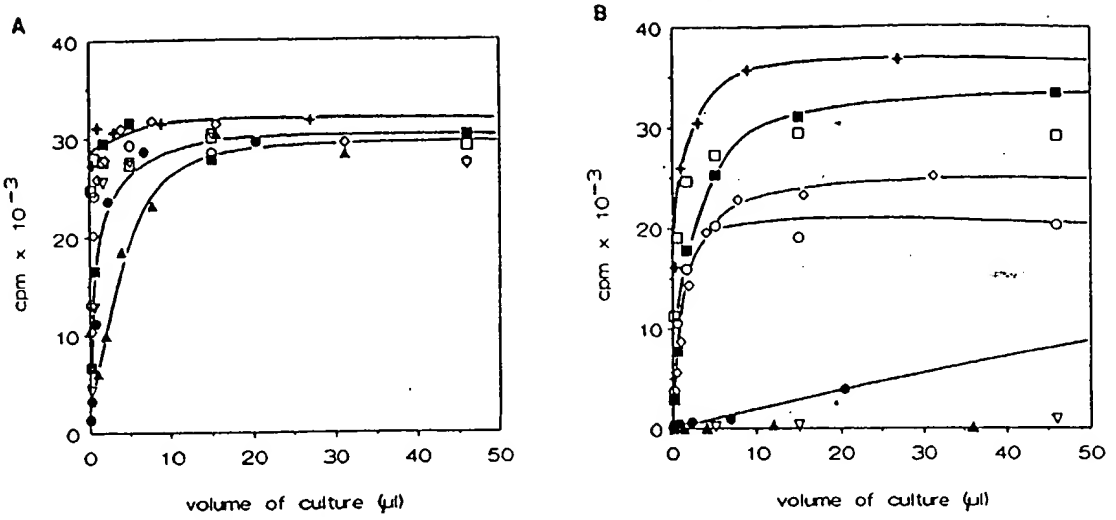


Fig. 3. Analysis of mutant CT-Bs with substitutions for Gly-33 by sandwich-SPRIA (A) and GM1-SPRIA (B). Extracts of *E. coli* strains carrying the following plasmids were tested: pMGJ19 wild type (+); pMGJ1903 Glu (▽); pMGJ1911 Ala (■); pMGJ1941 Val (●); pMGJ1943 Gln (○); pMGJ1910 Arg (□); pMGJ1949 Ile (▲); pMGJ1942 Lys (◇).

For each CT-B mutant the binding observed by S-SPRIA was directly proportional to the binding in GM1-SPRIA over an appropriate range of concentrations (Fig. 4; Olsvik *et al.*, 1983). The slopes of the lines in Fig. 4 provided quantitative measures of GM1-binding activity per unit of immunoreactive wild-type or mutant CT-B. Purified holotoxin, B pentamer or wild-type CT-B in crude extracts had a characteristic slope with an absolute value of 0.39 to 0.41. Relative binding was expressed as the slope for each mutant CT-B divided by the slope for wild-type CT-B (Table 2), and the relative binding for wild-type CT-B was assigned a value of 1. At the concentrations used for this analysis, extracts containing mutant proteins with Asp-33, Glu-33, Ile-33 or Leu-33 substitutions showed no binding to GM1. The relative binding value of the Val-33 mutant was 0.07, while Ser-33, Thr-33 and Gln-33 mutant proteins had relative binding values slightly less than that of the wild type (Table 2). Lys-33, Arg-33 and Ala-33 mutant proteins were indistinguishable from the wild type in terms of relative binding activity.

Effects of amino acid substitutions for tryptophan-88

Using the same strategy as for glycine-33 mutants, mutations at the Trp-88 codon were generated in pMGJ19, using the oligonucleotide Trp-88-NNN (Table 1). Transformants were screened by differential colony hybridization followed by RPIHA. Twenty-three mutants were identified by hybridization, and all were negative by RPIHA. Sequencing of these mutants demonstrated that 14 out of the possible 19 amino acid replacements and two termination mutants were obtained (Table 2). When

Table 2. Characterization of mutant CT-B clones.

Position substituted	MGJ no.	Amino acid	Codon	RPIHA[a] phenotype	Antigen titre[b]	Relative binding[c]
None	19	Wt[d]	Wt	+	700	1.0
Gly-33 (GGA)	1903	Glu	G A A	–	1000	0
	1950	Glu	G A G	–	ND[e]	–
	1948	Asp	G A T	–	5000	0
	1919	Ile	A T A	–	500	0.01
	1949	Ile	A T A	–	ND	–
	1954	Ile	A T T	–	ND	–
	1951	Leu	C T A	–	1250	0
	1941	Val	G T G	–	800	0.07
	1955	Val	G T A	–	ND	–
	1943	Gln	C A A	–	800	0.46
	1947	Ser	A G T	–	800	0.6
	1915	Thr	A C A	–	300	0.8

Table 2 continued.

Position substituted	MGJ no.	Amino acid	Codon	RPHA[a] phenotype	Antigen titre[b]	Relative binding[c]
	1911 (1914)	Ala	G C G	–	2500	1.0
	1942	Lys	A A A	–	2500	1.0
	1946	Lys	A G G	–	ND	+
	1910 (1917)	Arg	C G A	–	600	1.1
	1944	Arg	A G A	–	ND	+
	1945	Arg	C G G	–	ND	+
	1916	Ochre	T A A	–	NA[f]	NA
	1918	Opal	T G A	–	NA	NA
	1934	Gly	G G C	+	ND	ND
	1935	Gly	G G A	+	ND	ND
	1940	Gly	G G G	+	ND	ND
Trp-88	1922	Ile	A T T	–	130	0.6
(TGG)	1920	Leu	T T G	–	50	0.6
	1923	Gln	C A G	–	50	0.9
	1913 (1928)	Gln	C A A	–	ND	+
	1924	Val	G T G	–	35	1.1
	1938	His	C A T	–	20	1.3
	1933	Lys	A A G	–	10	0
	1908	Lys	A A A	–	ND	–
	1936	Asn	A A T	–	4	1.1
	1907	Glu	G A A	–	3	0
	1930	Met	A T G	–	<2	+
	1927	Pro	C C A	–	<2	NA
	1904	Ser	T C T	–	<2	NA
	1906	Ser	T C G	–	<2	NA
	1931	Ser	T C A	–	<2	NA
	1912	Gly	G G A	–	<2	NA
	1937	Gly	G G G	–	<2	NA
	1921	Thr	A C C	–	<2	NA
	1926	Arg	C G A	–	<2	NA
	1902	Opal	T G A	–	NA	NA
	1925	Ochre	T A A	–	NA	NA
Arg-35	1972	Asp	G A T	–	2000	1.8
(AGA)	1971	Glu	G A A	±	2000	1.9
	1973	Cys	T G C	±	30	+
	1966	His	C A C	+	2000	1.5
	1957	Gly	G G G	+	1500	1.4
	1960	Trp	T G G	+	1200	1.4
	1961	Ser	T C A	+	3000	1.1
	1964	Tyr	T A T	+	2000	0.7
	1969 (1956)	Ile	A T A	+	2000	1.0
	1968	Met	A T G	+	>256	+
	1963	Asn	A A C	+	>256	+
	1967	Gln	C A A	+	>256	+
	1962	Arg	C G A	+	ND	ND
	1965	Arg	A G G	+	ND	ND
	1959	Amber	T A G	–	NA	NA
	1970	Opal	T G A	–	NA	NA
Lys-34	1102	Gly	G G C	±	2200	+
(AAA)	1103	Ala	G C C	±	>256	+
	1104	Ser	A G C	±	ND	+
	1105	Cys	T G C	–	10	+
	1108	Val	G T C	±	ND	+
	1109	Ile	A T C	±	7000	+
	1110	Asp	G A C	±	8000	+
	1112	Tyr	T A C	+	>256	+
	1113	Asn	A A C	+	>256	+

a. +, wild-type halo; –, no halo; ±, non-wild-type halo.
b. Dilution of extract giving 50% maximal counts by S-SPRIA.
c. Ratio of slopes of lines from GM1 binding plotted against S-SPRIA binding, for mutant divided by wild-type. + or – quantitative assays not performed but mutant CT-B bound (+) or did not bind (–) in GM1-SPRIA.
d. Wild type.
e. Not determined.
f. Not applicable.

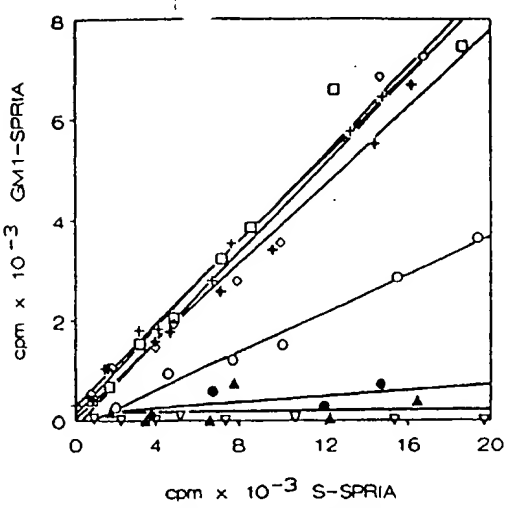


Fig. 4. Relative GM1-binding activity of selected mutant CT-Bs with substitutions for Gly-33. Each periplasmic extract was first diluted to give a signal of approximately 20000 c.p.m. by S-SPRIA, and eight additional dilutions over a 20-fold range were prepared and assayed by S-SPRIA and GM1-SPRIA. The results for the two assays for each dilution were plotted against each other. Extracts of *E. coli* TG1 carrying the following plasmids were tested: pMGJ1903 Glu (▽); pMGJ1941 Val (●); pMGJ1949 Ile (▲); pMGJ1943 Gln (○); pMGJ1910 Arg (□); pMGJ19 wild type (+). In addition, purified CT (+) and CT-B (◇) were included as controls.

tested by S-SPRIA (Fig. 5A and Table 2), none of the Trp-88 substitution mutants produced as much immunoreactive CT-B protein as the wild type. The Ile-88 mutant produced the greatest amount of immunoreactive toxin (19% of wild type, Fig. 5A). Mutants that produced lower, but still detectable, amounts of immunoreactive CT-B included Leu-88, Gln-88, Val-88, His-88, Lys-88, Asn-88, and Glu-88. Mutants that produced no detectable immunoreactive CT-B included Met-88, Pro-88, Ser-88, Gly-88, Thr-88 and Arg-88.

Although the mutant CT-Bs with substitutions for Trp-88 were produced in smaller amounts than wild-type CT-B, their relative binding to GM1 was comparable to wild-type CT-B, except for Lys-88 and Glu-88, which failed to bind (Fig. 5B, Table 2). Therefore, a positively charged residue or a negatively charged residue at position 88 abolished receptor-binding activity. The observation that the relative GM1-binding activity of mutant CT-B proteins containing Ile-88, Leu-88, Gln-88, Asn-88 or His-88 was similar to that of the wild type indicates that Trp-88 is not essential for binding of CT-B to GM1. The dramatic effect of all substitutions for Trp-88 on the amount of immunoreactive toxin suggests that Trp-88 is important for establishing or maintaining the native conformation of CT-B.

Effects of amino acid substitutions for arginine-35

Similar strategies were used to obtain and characterize substitutions for arginine-35 of *ctxB*, in pMJG19, using the oligonucleotide Arg-35-NNN (Table 1). Among 50 transformants tested, only three had mutant phenotypes by RPIHA, whereas 17 had mutant genotypes revealed by colony hybridization with the wild-type oligonucleotide probe. Most substitutions for Arg-35, therefore, do not produce mutant phenotypes by RPIHA. The three clones with no halo or with abnormal haloes were identified as Cys-35, Glu-35 and Asp-35 substitutions (Table 2). The Cys-35 mutant gave a small turbid halo and the Glu-35 mutant gave a normal-sized but turbid halo. Only the

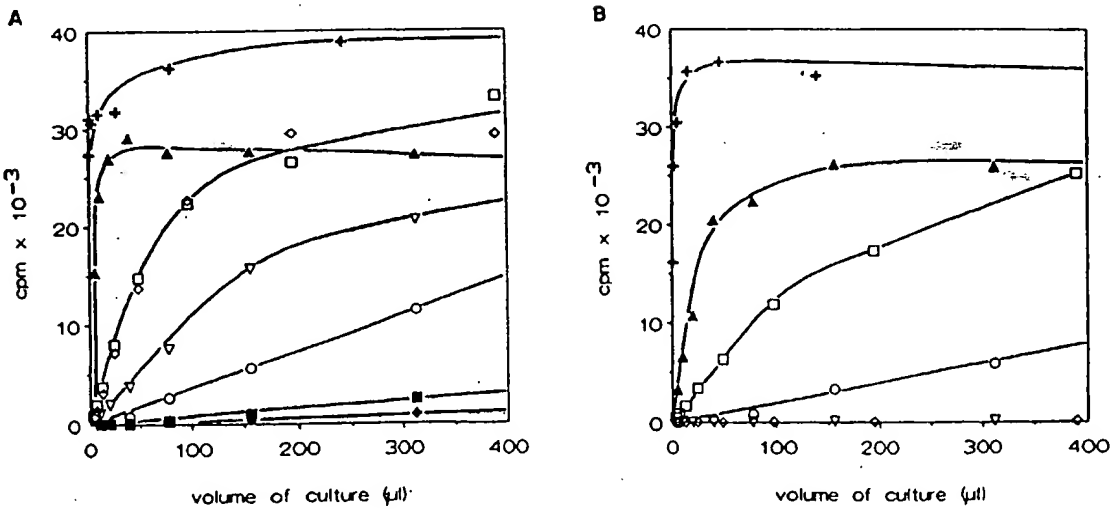


Fig. 5. Analysis of mutant CT-Bs with substitutions for Trp-88 by sandwich-SPRIA (A) and GM1-SPRIA (B). Extracts of *E. coli* strains carrying the following plasmids were tested: pMGJ19 wild type (+); pMGJ1920 Ile (▲); pMGJ1933 Lys (◇); pMGJ1938 His (□); pMGJ1907 Glu (▽); pMGJ1904 Ser (◆); pMGJ1921 Thr (■); pMGJ1936 Asn (○).

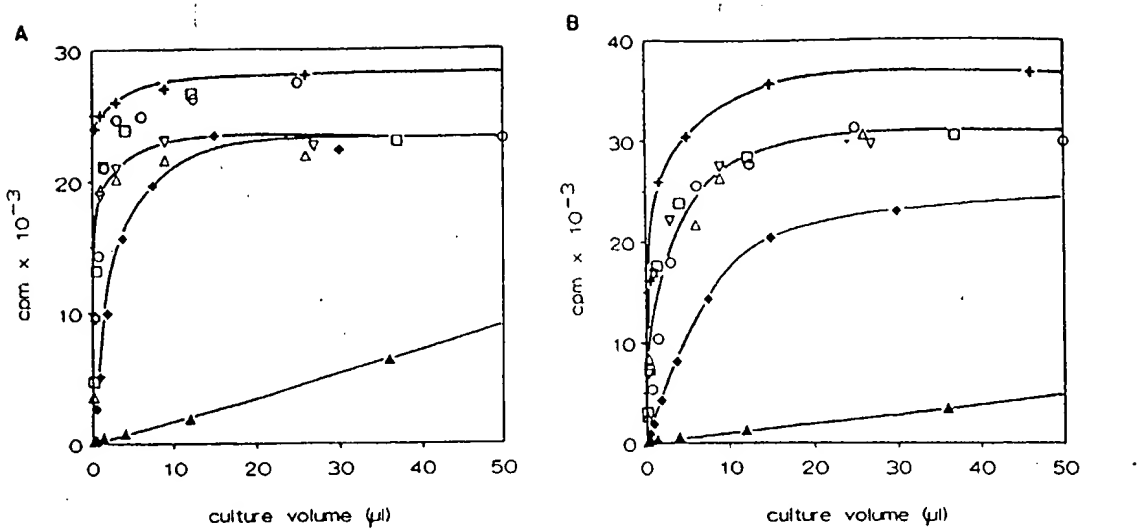


Fig. 6. Analysis of mutant CT-Bs with substitutions for Arg-35 by sandwich-SPRIA (A) and GM1-SPRIA (B). Extracts of *E. coli* strains carrying the following plasmids were tested: pMGJ19 wild type (+); pMGJ1971 Asp (Δ); pMGJ1972 Glu (▽); pMGJ1973 Cys (▲); pMGJ1957 Gly (○); pMGJ1966 His (□); pMGJ1960 Trp (◆).

Asp-35 mutant gave no halo. The 14 other mutants identified by hybridization included two wild-type CT-Bs with alternative arginine codons, two termination mutants, and 10 missense mutations representing nine other amino acid replacements. All of the missense mutants tested, except one, produced high levels of immunoreactive toxin that also bound to GM1 (Figs 6A, 6B). The Cys-35 mutant produced a lower level of immunoreactive CT-B. Most mutants tested, including Glu-35 and Asp-35 had relative GM1-binding values that were slightly higher than that of wild-type CT-B.

Effects of amino acid substitutions for lysine-34

Ludwig *et al.* (1985) showed that one or more lysines of the B subunit were involved in the interaction between CT-B and GM1. Because of the proximity of Lys-34 to Gly-33, we tested whether substitutions at Lys-34 would affect GM1-binding activity. Oligonucleotide Lys-34-NNC (Table 1) was designed to replace the Lys-34 codon in pMGJ11 with a codon specifying any of 15 amino acids (fixing the third position at C excluded recovery of the wild-type amino acid Arg, Met, Glu, Trp or termination codons). Twenty transformants screened by RPIHA were sequenced. Among five clones that were negative by RPIHA, two were frameshift mutations, two were IS*1* insertions at the same position within the B gene, and one was a substitution mutant (Cys-34). Eleven mutants produced normal-sized but turbid haloes, and represented six different amino acid substitutions (Gly-34, Ala-34, Ser-34, Val-34, Ile-34 and Asp-34). Two transformants with wild-type haloes, randomly selected and sequenced, had substitutions of Tyr and Asn for Lys-34. All of the mutant CT-Bs tested produced normal high levels of immunoreactive CT-B, with the exception of Cys-34. The phenotype of this mutant was similar to that of the Cys-35 mutant. All of the mutants tested bound to GM1 in the GM1-SPRIA (data not shown), but quantitative titrations were not performed for the determination of relative binding activity. The effect of negatively charged or hydrophobic amino acid substitutions for Lys-34 was very different from the effects of the corresponding substitutions for Gly-33, and Lys-34 did not appear to be involved in the interaction of CT-B with GM1.

Interaction of mutant toxins with sheep red blood cells (SRBCs)

Most of the mutant CT-B proteins retained the ability to bind to GM1 in the solid-phase immunoassay, yet failed to cause haemolysis of SRBCs in the RPIHA. As a direct test of the ability of selected mutant B subunits to bind to SRBCs, extracts were adsorbed with SRBCs and the supernatants were tested for unadsorbed CT-B (Fig. 7). All of the selected mutants with RPIHA-negative phenotypes failed to bind to SRBCs, in contrast to the extensive binding of wild-type CT-B (>80%). Previous reports demonstrated that the susceptibility of target cells to cholera toxin could be increased by incubating the cells with GM1 prior to exposing the cells to toxin (Cuatrecasas, 1973). We therefore tested whether the susceptibility of SRBCs to immune haemolysis by mutant CT-Bs could be enhanced by pretreating the SRBCs with GM1. The

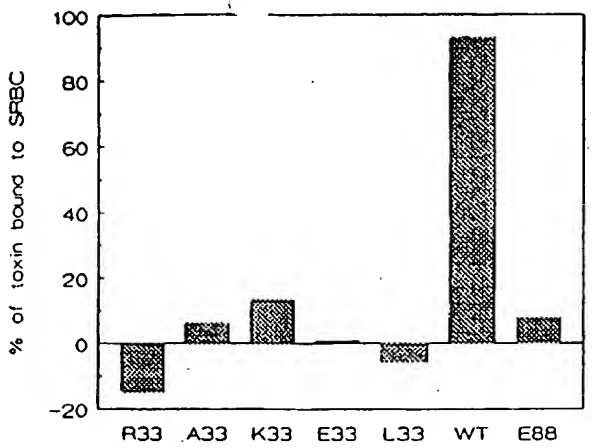


Fig. 7. Lack of binding of mutant B subunits to SRBCs. Periplasmic extracts were diluted to concentrations that gave a signal of 5000 to 15000 c.p.m. by S-SPRIA, and 100 µl aliquots were incubated with 20 µl of washed SRBCs or phosphate-buffered saline (PBS) for 1 h. SRBCs were pelleted, and the supernatants were assayed by S-SPRIA. The percentage of toxin bound was calculated as:

$$\frac{(\text{c.p.m. in PBS supernatant} - \text{c.p.m. in SRBC supernatant}) \times 100}{\text{c.p.m. in PBS supernatant}}$$

GM1-sensitized SRBCs became sensitive to immune haemolysis by the Ala-33 mutant CT-B, but remained resistant to immune haemolysis by all other mutant CT-Bs with substitutions for Gly-33.

Formation and toxicity of holotoxins containing mutant CT-Bs

To test the effect of *ctxB* mutations on holotoxin formation and activity, we introduced selected *ctxB* clones into *E. coli* carrying cloned *ctxA* and *toxR* genes on compatible plasmids. Crude extracts of these strains were tested for immunoreactivity by modified S-SPRIA and GM1-SPRIA using monoclonal anti-CT-A antibodies, and for toxicity by using the mouse Y1 adrenal cell assay (Table 3).

Most CT-B mutations, with the exceptions of Glu-35 and Asp-35, did not affect the ability of the mutant CT-Bs to associate with the A subunit to form immunoreactive holotoxin molecules, detected by the modified SPRIAs. The amounts of holotoxin antigen were proportional to the amounts of immunoreactive CT-B detected in previous assays of clones that produced only CT-B.

In most cases the relative toxicities of mutant holotoxins correlated well with the GM1-binding activities of their mutant CT-Bs. Holotoxins containing mutant CT-Bs that failed to bind to GM1 (Glu-33, Asp-33, Ile-33, Leu-33 and Val-33) were non-toxic. Most strains carrying mutant CT-Bs that bound to GM1 produced holotoxins that were of comparable toxicity to wild-type CT. The principal exceptions were the Arg-33 and Lys-33 mutants of CT-B that bound to GM1 as well as wild-type CT-B but formed holotoxins that showed no toxicity (Lys-33) or reduced toxicity (Arg-33). Holotoxin formed with Gln-33 CT-B had slightly reduced toxicity, consistent with the decreased GM1-binding activity of the Gln-33 CT-B. Only a small amount of holotoxin was formed with Ile-88 CT-B, but its relative toxicity was equivalent to that of wild-type CT. The other Trp-88 substitution mutants produced insufficient levels of CT-B to be detected in this assay. Holotoxin formed with Asp-34 mutant CT-B was produced in normal amounts and had wild-type toxicity. Holotoxins containing Glu-35 and Arg-35 mutant CT-Bs were significantly less stable than other mutant holotoxins; their toxicity and holotoxin immunoreactivity were only detectable in freshly prepared extracts, and could not be detected two days later (Table 3), although the mutant B subunits were stable and were produced in wild-type amounts.

Discussion

The production of mutant CT-Bs by oligonucleotide mutagenesis allowed us to determine the relative importance of several amino acids for the function of the B subunit of cholera toxin. Substitution of serine for Cys-9, Cys-86 or both prevented formation of the disulphide link between residues 9 and 86, and blocked formation of stable and immunoreactive CT-B. This confirms the observations of Ludwig *et al.* (1985) that reduction and carboxymethylation of these cysteine residues destroys antigenicity of CT-B, and of Hardy *et al.* (1988), who showed that reduced LT-B monomers were rapidly degraded *in vivo* in *E. coli*. This may also explain why the cysteine replacements for Lys-34 or Arg-35 were produced at low levels, since aberrant crosslinking of Cys-34 or Cys-35 to either Cys-9 or Cys-86 would cause a dramatic change in the structure of the mutant CT-B.

Substitution of aspartate for glycine at position 33 in LTp-I abolishes its ability to bind to GM1 (Tsuji *et al.*, 1985). This observation indicates that Asp-33 is incompatible with GM1-binding ability but does not establish whether or not glycine is essential. Our results with CT-B showed that several amino acids can substitute for Gly-33 without affecting GM1-binding ability. We conclude, therefore, that Gly-33 is not required for binding of CT-B to GM1. Substitution of a negatively charged or large hydrophobic residue for Gly-33 eliminated binding to GM1. Comparison of the effects of various substitutions enabled us to assess (separately) the contributions of size, charge, and hydrophobicity of the amino acid side-chain at residue 33. Among the residues with hydrophobic side-chains, Ala-33 was wild-type in its GM1-binding phenotype, but Val-33, Leu-33 and Ile-33 caused progressively decreasing GM1-binding activity. Comparison of the Glu-33 and Gln-33 mutants showed the importance of the

Table 3. Toxicity of holotoxins containing mutant CT-Bs.

Position substituted	MGJ no.	Amino acid	Toxicity (U ml^{-1})	Toxin antigen (μg ml^{-1})	Specific toxicity (U μg^{-1} antigen)	Ratio to wild type
None	19	Wt	0.80×10^3	1.50	0.53×10^3	1
Gly-33	1903	Glu	<10	8	<1.25	<0.002
	1948	Asp	<10	6	<1.67	<0.003
	1919	Ile	<10	0.25	<4.0	<0.08
	1951	Leu	<10	1.6	<6.3	<0.01
	1941	Val	<10	0.8	<12	<0.02
	1942	Lys	<10	0.5	<20	<0.04
	1946	Lys	<10	0.375	<30	<0.06
	1910	Arg	1.28×10^3	4.5	0.28×10^3	0.5
	1944	Arg	0.24×10^3	1.3	0.18×10^3	0.35
	1947	Ser	2.52×10^3	2.8	0.90×10^3	1.7
	1943	Gln	0.32×10^3	1.25	0.26×10^3	0.5
	1911	Ala	0.64×10^3	0.75	0.85×10^3	1.6
	1915	Thr	1.28×10^3	1.25	1.02×10^3	1.9
Trp-88	1922	Ile	10	0.012	0.83×10^3	1.5
Arg-35	1971	Glu	40 (<10)[a]	0.2 (0)	0.20×10^3	0.4
	1972	Asp	20 (<10)	0.06 (0)	0.33×10^3	0.6
	1960	Trp	0.20×10^3	0.44	0.45×10^3	0.85
	1964	Tyr	0.80×10^3	3.8	0.21 ± 10^3	0.4
	1963	Asn	2.60×10^3	8.0	0.32×10^3	0.6
	1966	His	0.16×10^3	0.28	0.57×10^3	1.0
Lys-34	1110	Asp	0.32×10^3	0.50	0.64×10^3	1.2

a. Values in parentheses represent assays performed 48 h after preparation of extracts.

negative charge of the carboxyl group in interfering with receptor binding. The positively charged residues arginine and lysine did not interfere with the GM1-binding activity of the mutant CT-Bs in spite of the size and length of the side-chains. We conclude that size alone is probably less important than negative charge or hydrophobicity in determining the effects of substitution for Gly-33 on GM1-binding activity of CT-B.

Substitutions at position 88, unlike those at 33, led to markedly decreased levels of immunoreactive protein detected. No amino acid substitution had a wild-type phenotype, and half of the substitution mutants made no detectable CT-B. Replacement of Trp-88 with the hydrophobic residue isoleucine, leucine or valine, or the polar residue glutamine, resulted in production of the largest amounts of immunoreactive CT-B. Other mutants produced lower amounts of mutant protein or none at all. For each amino acid substitution of Trp-88, a variable but reproducible proportion of the mutant protein was capable of being secreted and attaining an immunoreactive conformation. These mature mutant CT-Bs were as stable as wild-type CT-B (data not shown). The defect in production of these mutant CT-Bs could occur at any step in the pathway from gene to mature protein. Missense mutations are unlikely to have drastic effects on transcription or translation. The cytoplasmic CT-B mutant precursor polypeptide could be degraded prior to or during secretion across the inner membrane, or could adopt a conformation incompatible with secretion (Randall and Hardy, 1986; MacIntyre and Henning, 1990). Alternatively, the mutant polypeptides could be more susceptible to proteolysis in the periplasm or less able to fold into the wild-type conformation. The mutations appear to affect the proportion of monomers that complete these processes but do not seem to affect the stability of the immunoreactive mutant CT-B that is formed. Similar effects of substitution for a tryptophan residue of aspartate aminotransferase were noted by Mattingly, Jr, and Martinez-Carrion (1990), who showed greatly reduced yields of mutant proteins when Trp-140 was substituted with Phe or Gly. Retention of a significant level of catalytic activity by the Phe-140 mutant enzyme suggested that the importance of the Trp residue was mainly as a structural element. The low yield but near wild-type stability of the purified mutants also led to the suggestion of additional roles for the Trp residue in efficient folding and assembly of the nascent enzyme *in vivo*. All of the Trp-88 mutant CT-Bs showed near wild-type relative binding values for GM1, except for Lys-88 and Glu-88 mutants that did not bind to GM1. We conclude that Trp-88 has an important role in determining the tertiary or quaternary structure of CT-B. Although Trp-88 is not required for binding of GM1

Table 4. Plasmids, vectors and constructs.

Plasmid	Marker	Replicon	Promoter	Genotype	Source
pKS⁻	Ap	ColE1	*lac*		Stratagene
pKS⁺	Ap	ColE1	*lac*		Stratagene
M13mp19	–	M13	*lac*		Yanisch-Perron *et al.* (1985)
pACYC184	Cm Tc	p15a	*tet*		Chang and Cohen (1978)
pRK404	Tc	IncP	*lac*		Ditta *et al.* (1985)
pVM25	Cm	p15a	*tet*	*toxR*⁺	Miller and Mekalanos (1984)
pCVD30	Cm Ap	ColE1	ct	*ctxA*⁻*B*⁺	Kaper *et al.* (1986)
pCVD14	Cm Ap	ColE1	ct	*ctxA*⁺*B*⁺	Kaper *et al.* (1986)
pMGJ8	Ap	ColE1	ct	*ctxB*⁺	This study
pMGJ19	Ap	ColE1	ct	*ctxB*⁺	This study
pMGJ11	Ap	ColE1	*lac*, ct	*ctXB*⁺	This study
pMGJ14	Cm	p15a	ct	*ctxA*⁺	This study
pMGJ40	Tc	IncP	*tet*	*toxR*⁺	This study

to CT-B, its location in or near the actual receptor binding site is suggested by the effects of binding on tryptophan fluorescence (de Wolf *et al.*, 1981), the results of chemical modification studies (Ludwig *et al.*, 1985), and the phenotypes of the Lys-88 and Glu-88 mutants reported here.

All of the observed amino acid substitutions for Arg-35, except Cys-35, produced wild-type levels of immunoreactive CT-B. Most substitutions were compatible with receptor binding and binding to SRBCs by RPIHA, demonstrating that Arg-35 is not critically involved in binding to GM1. Negatively charged substitutions (Glu and Asp) did affect binding to SRBCs by RPIHA but not to purified GM1 by SPRIA. This supports the conclusions of Ludwig *et al.* (1985) that modification of any single arginine residue, specifically Arg-35, did not affect receptor binding. Our data also eliminate any requirement for Lys-34 in the GM1 binding activity of CT-B. One or more of the remaining eight lysine residues could still be involved in binding of CT-B to GM1, as proposed by Ludwig *et al.* (1985).

Residues 33, 34, and 35 of mature CT-B are able to accommodate a wide variety of amino acid substitutions without significant effects on the level of CT-B produced or its immunoreactivity, implying that these residues are located in a region of the toxin molecule that has few structural constraints on the type of side-chain present. This is in contrast with the adverse effects of most substitutions for Trp-88 on production of immunoreactive CT-B.

We noted that *in vitro* binding of mutant CT-Bs to GM1 did not correlate with binding to SRBCs. These observations suggest that the binding sites for CT-B on SRBCs that mediate complement-mediated immune haemolysis are not GM1. It is known that CT and the related LTs will bind to receptors other than GM1, although with reduced affinity (Fukuta *et al.*, 1988; Griffiths *et al.*, 1986). Because rat RBCs are known to be rich in GM1 and fucosyl-GM1 (Iwamori *et al.*, 1983) and both can function as receptors for CT, we tested them as indicator cells in the RPIHA. Results were identical to those obtained with SRBCs, in that all Gly-33 mutant extracts were unable to form a halo on either cell type. Iwamori *et al.* (1983) also detected a cryptic form of GM1 on the rat cell surface that failed to react with anti-GM1 antibodies, but CT was still able to bind to the rat erythrocytes (Iwamori *et al.*, 1985). It will be of interest to determine the nature of the active receptor for CT-B on SRBCs that mediate immune haemolysis in our RPIHAs.

Only two of the mutant CT-Bs tested (Glu-35 and Asp-35) were affected in terms of their ability to associate with CT-A to form immunoreactive CT holotoxin. Mutants with negatively charged residues at position 35 formed small amounts of unstable holotoxin. This was not because of instability of the mutant B subunit, which remained detectable over time in amounts comparable to wild-type CT-B. This finding suggests that residue 35 is located in a region of the B subunit that interacts with the A subunit in holotoxin and also that negatively charged residues at position 35 interfere with holotoxin assembly and stability.

Substitution of Gly-33 with the positively charged residue arginine or lysine did not affect the formation of holotoxin but reduced the relative toxicity of the mutant holotoxin. Decreased toxicity is not likely to be caused by decreased receptor binding, since these mutant holotoxins containing Lys-33 or Arg-33 mutant CT-Bs bound as well as wild-type CT to purified GM1 on polystyrene immunoassay plates. It is possible that these mutants are defective in a step required for toxicity that occurs after binding to the cells, e.g. a conformational change in the bound holotoxin that is believed to facilitate release of and penetration of the A subunit into and through the plasma membrane (Surewicz *et al.*, 1990).

All of the studies reported here were performed with crude extracts of mutant CT-Bs or mutant holotoxins in specific immunoassays or bioassays. The results provide important information about the roles of Cys-9, Gly-33, Lys-34, Arg-35, Cys-86 and Trp-88 for the structure and

function of CT-B. Future studies with selected purified mutant CT-Bs should provide additional information about the mechanism of the GM1 binding, assembly of CT-B pentamer and CT holotoxin, and interactions of CT with plasma membranes of specific target cells. Correlation of our observations with the three-dimensional structure of CT-B is also important but cannot be performed until the molecular structural analysis of CT or LT has been accomplished at a higher level of resolution than is currently available (Tsuji *et al.*, 1989; Spangler and Westbrook, 1989; Ribi *et al.*, 1988).

Experimental procedures

Bacterial strains and plasmids

E. coli strains TG1 (*thi, hsdD5, glnV44,* Δ(*lac–pro*), F′*traD36, proAB, lacI*^{q}, *lacZ*ΔM15; Amersham International PLC) or DH5α (*endA1, hsdR17, glnV44, thi1, recA1, gyrA, relA1,* Δ(*lacZYA–argF*)U169 Φ80d*lacZ*ΔM15; BRL) were used as the hosts for the plasmids listed below, except for the preparation of uracil-containing single-stranded (ss) DNA when CJ236 (*dut, ung, thi, relA,* pCJ105, Cm^R; BioRad) was used. Cloning vectors used are listed in Table 4.

DNA techniques and clone constructions

Mini-prep plasmid DNA was isolated using an alkaline lysis method (Morelle, 1989). Restriction and other enzymes were used as described by the manufacturers, and digestions were analysed on 0.8% agarose gels. Plasmid constructs are listed in Table 4. Plasmid pMGJ8 consists of a 1.5 kb *Hpa*II–*Hae*III fragment from pCVD30 cloned into pKS$^-$ in the opposite orientation to the *lac* promoter. Plasmid pMGJ11 consists of a 0.9 kb *Hpa*II–*Nsp*CI *ctxB*-containing fragment from pCVD30 in pKS$^-$, under the control of the *lac* promoter. Plasmid pMGJ19 consists of the same fragment in pKS$^+$ in the opposite orientation to the *lac* promoter. The *ctxA*-producing clone pMGJ14 was constructed by cloning a filled-in 1.6 kb *Acc*I fragment from pCVD14 into the filled-in *Bam*HI site of pACYC184. The *toxR*-producing clone pMGJ40 was constructed by cloning a 1.6 kb *Hin*dIII–*Bam*HI fragment from pVM25 into pRK404. DNA was transformed into cells made competent by the method of Hanahan (1983).

Oligonucleotide-directed mutagenesis and DNA sequencing

We determined the sequence of the *ctxB* gene of pCVD14, one of the two *ctx* operons from the Classical biotype *V. cholerae* strain O395 (Jobling *et al.*, 1991a) and showed it to differ at several positions from the sequence for the *ctx* operon from *V. cholerae* 2125 (Mekalanos *et al.*, 1983). Oligonucleotides were designed on the basis of our sequence; the DNA sequence coded for a protein with a predicted amino acid sequence identical to the mature CT-B peptide from *V. cholerae* 569B (Lai, 1977), as corrected by assigning aspartate instead of asparagine to amino acids 22 and 70 (Takao *et al.*, 1985). Oligonucleotides were made with an Applied Biosystems DNA synthesizer or purchased from GDI. Mutagenesis was performed as described in the BioRad *Muta-Gene Manual* but using primer to template ratios of 5:1 and an annealing temperature of 15°C (Goff *et al.*, 1987). Uracil-containing ssDNA template was prepared from CJ236(pMGJ19) using helper phage M13K07 (Pharmacia-LKB) or R408 (Russel *et al.*, 1986). The products of oligonucleotide-primed DNA synthesis reactions were transformed into *E. coli dut*$^+$, *ung*$^+$ strain TG1(pVM25), and chloramphenicol- and ampicillin-resistant (Cm^R, Ap^R) transformants were selected. Mutant colonies were detected by differential hybridization in colony blots using [^{32}P]-5′ end-labelled wild-type oligonucleotide as probe (*Amersham Mutagenesis Manual*, Amersham International PLC). Dideoxy DNA sequencing was done using *ctx*-specific primers and T7 DNA polymerase (Sequenase, USB) and reactions were analysed on buffer gradient 6 or 8% polyacrylamide–urea gels (Sheen and Seed, 1988).

Assays for CT-B and CT

Transformants were screened for production of CT-B by observing whether haloes were formed in a radial passive immune haemolysis assay (RPIHA, Bramucci and Holmes, 1978) using a sheep erythrocyte overlay on LA plates (Fig. 2). In addition, extracts were prepared from 30- to 50-fold concentrated suspensions of bacteria by treatment with polymyxin B (Finkelstein and Yang, 1983) or by sonication of 10-fold concentrated bacterial cultures. Quantification of immunoreactive CT-B was achieved using a modified sandwich solid-phase radioimmunoassay (S-SPRIA; Bramucci *et al.*, 1981) and GM1-solid-phase radioimmunoassay (GM1-SPRIA; Holmes and Twiddy, 1983). The published method for S-SPRIA was modified by substituting purified anti-CT IgG from goat serum for affinity-purified equine serum, and using monospecific rabbit anti-CT-B as the second antibody. CT holotoxin was quantified using a monoclonal anti-CT-A antibody (21B11; Holmes and Twiddy, 1983) to detect antigen bound to GM1 or goat-anti-CT-coated plates, and bound monoclonal antibody was detected by sequential incubation with rabbit anti-mouse IgG and [^{125}I]-labelled goat anti-rabbit IgG. Biological activity was determined by assessing rounding of mouse Y1 adrenal cell monolayers after exposure to toxin (Maneval *et al.*, 1980).

Protein labelling

Plasmid gene products were specifically labelled *in vivo* with [^{35}S]-methionine using the inducible T7 RNA polymerase system. One-millilitre cultures were grown and treated as described (Tabor and Richardson, 1985) and labelled with 10 μCi of [^{35}S]-methionine for 60 min. The cells were pelletted in a microfuge, resuspended in 1/10th volume of 10 mM Tris (pH 8) containing 2 mg ml^{-1} polymyxin B for 10 min at 37°C, and then pelletted. The supernatant (periplasmic extract) was mixed with an equal volume of 2× sample buffer without β-mercaptoethanol, the cell pellet was boiled in sample buffer with β-mercaptoethanol, and both were analysed by PAGE using 16%T–3%C discontinuous tricine-buffered SDS–PAGs (Schagger and Jagow, 1987). The gel was fixed, infiltrated with fluor (Jen and Thatch, 1982), and dried. Autoradiograms were made with intensifying screens using Kodak X-Omat AR film exposed at −70°C.

Media and chemicals

2YT broth (Bankier and Barrel, 1983) and LB plates (Miller, 1972) were used for routine bacterial cultures at 37°C. Antibiotics were

added where required, at the following concentrations: ampicillin, 50 μg ml $^{-1}$; chloramphenicol, 25 μg ml^{-1}; tetracycline, 10 μg ml^{-1}.

Acknowledgements

This work was supported, in part, by protocol number RO7301 from the Uniformed Service University of the Health Sciences. The opinions and assertions contained herein are the private views of the authors and should not be construed as official or as necessarily reflecting the views of the University or the Department of Defense.

References

Bankier, A.T., and Barrel, B.G. (1983) Shotgun DNA sequencing. *Tech Life Sci* B5-08: 1–34.

Betley, M.J., Miller, V.L., and Mekalanos, J.J. (1986) Genetics of bacterial enterotoxins. *Annu Rev Microbiol* 40: 577–605.

Bramucci, M.G., and Holmes, R.K. (1978) Radial passive immune hemolysis assay for detection of heat-labile enterotoxin produced by individual colonies of *Escherichia coli* or *Vibrio cholerae*. *J Clin Microbiol* 8: 252–255.

Bramucci, M.G., Twiddy, E.M., Baine, W.B., and Holmes, R.K. (1981) Isolation and characterization of hypertoxinogenic (*htx*) mutants of *Escherichia coli* KL320(pCG86). *Infect Immun* 32: 1034–1044.

Chang, A.C.Y., and Cohen, S.N. (1978) Construction and characterization of amplifiable multicopy cloning vehicles derived from the p15A cryptic miniplasmid. *J Bacteriol* 134: 1141–1156.

Cuatrecasas, P. (1973) Gangliosides and membrane receptors for cholera toxin. *Biochem* 12: 358–3566.

de Wolf, M.J.S., Fridkin, M., and Kohn, L.D. (1981) Tryptophan residues of cholera toxin and its A and B promoters. *J Biol Chem* 256: 5481–5488.

Ditta, G., Schmidhauser, T., Yakobson, E., Lu, P., Liang, W.-W., Finlay, D.R., Guiney, D., and Helinski, D.R. (1985) Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. *Plasmid* 13: 149–153.

Duffy, L.K., and Lai, C.Y. (1979) Involvement of arginine residues in the binding site of cholera toxin B subunit. *Biochem Biophys Res Commun* 91: 1005–1010.

Field, M.F., Mrinalini, C.R., and Chang, E.B. (1989) Intestinal electrolyte transport and diarrheal disease. *New Engl J Med* 321: 879–883.

Finkelstein, R.A., and Yang, Z.S. (1983) Rapid test for identification of heat-labile enterotoxin-producing *Escherichia coli* colonies. *J Clin Microbiol* 18: 23–28.

Finkelstein, R.A., Burks, M.F., Zupon, A., Dallas, W.S., Jacob, C.O., and Ludwig, D.S. (1987) Epitopes of the cholera family of enterotoxins. *Rev Inf Dis* 9: 544–561.

Fishman, P.H., Moss, J., and Osborne, J.C. (1978) Interaction of choleragen with oligosaccharide of ganglioside GM1 - evidence for multiple oligosaccharide binding sites. *Biochem* 17: 711–716.

Fukuta, S., Magnani, J.F., Twiddy, E.M., Holmes, R.K., and Ginsberg, V. (1988) Comparison of the carbohydrate-binding specificities of cholera toxin and *Escherichia coli* heat-labile enterotoxins LTh-I, LT-IIa and LT-IIb. *Infect Immun* 56: 1748–1753.

Goff, S.A., [illegible]t, M., Russell, S.R., and Dice, J.F. (1987) Efficient saturation mutagenesis of a pentapeptide coding sequence using mixed oligonucleotides. *DNA* 6: 381–388.

Griffiths, S.L., Finkelstein, R.A., and Critchley, D.R. (1986) Characterization of the receptor for cholera toxin and *Escherichia coli* heat-labile toxin in rabbit intestinal brush borders. *Biochem* 238: 313–322.

Guth, B.E., Pickett, C.L., Twiddy, E.M., Holmes, R.K., Gomes, T.A.T., Lima, A.A.M., Guerrant, R.L., Franco, B.D.G.M., and Tabulsi, L.R. (1986) Production of type II heat-labile enterotoxin by *Escherichia coli* isolated from food and human feces. *Infect Immun* 59: 587–589.

Hanahan, D. (1983) Studies on the transformation of *Escherichia coli* with plasmids. *J Mol Biol* 166: 557–580.

Hardy, S.J.S., Holmgren, J., Johansson, S., Sanchez, J., and Hirst, T.R. (1988) Coordinated assembly of multisubunit proteins: oligomerization of bacterial enterotoxins *in vivo* and *in vitro*. *Proc Natl Acad Sci USA* 85: 7109–7113.

Holmes, R.K., and Twiddy, E.M. (1983) Characterization of monoclonal antibodies that react with unique and cross-reacting determinants of cholera enterotoxin and its subunits. *Infect Immun* 42: 914–923.

Holmes, R.K., Twiddy, E.M., and Pickett, C.L. (1986) Purification and characterization of heat-labile enterotoxin of *Escherichia coli*. *Infect Immun* 53: 464–473.

Iida, T., Tsuji, T., Honda, T., Miwatani, T., Wakabayashi, S., Wada, K., and Matsubara, H. (1989) A single amino acid substitution in B subunit of *Escherichia coli* enterotoxin affects its oligomer formation. *J Biol Chem* 264: 14065–14070.

Iwamori, M., Shimomura, J., Tsujyuhara, S., Mogi, M., Ishizaki, S., and Nagai, Y. (1983) Differential reactivities of fucosyl-GM1 and GM1 gangliosides on rat erythrocyte membranes revealed by analysis with antifucosyl-GM1 and GM1 antisera. *J Biochem* 94: 1–10.

Iwamori, M., Shimomura, J., and Nagai, Y. (1985) Specific binding of cholera toxin to rat erythrocytes revealed by analysis with a fluorescence-activated cell sorter. *J Biochem* 97: 729–735.

Jen, G., and Thatch, R.E. (1982) Inhibition of host translation in encephalomyocarditis infected L cells. A novel mechanism. *J Virol* 43: 250–261.

Jobling, M.G., Marcus, H., and Holmes, R.K. (1991a) Genetic analysis of function and secretion of cholera toxin. In *Advances in Research on Cholera and Related Diarrheas*. Vol. 8. Tokyo: KTK Scientific Publishers, in press.

Jobling, M.G., Connell, T.D., and Holmes, R.K. (1991b) Molecular genetic analysis of binding activity of B subunits of enterotoxins of the *V. cholerae*/*E. coli* family. In *Advances in Research on Cholera and Related Diarrheas*. Vol. 10. Tokyo: KTK Scientific Publishers, in press.

Kaper, J.B., Lockman, H., Baldini, M.M., and Levine, M.M. (1986) Development of live oral cholera vaccine candidates through recombinant DNA techniques. In *Advances in Research on Cholera and Related Diarrheas*. Vol. 3. Kuwahara, S., and Pierce, N. (eds). Tokyo: KTK Scientific Publishers, pp. 181–191.

Lai, C.Y. (1977) Determination of primary structure of cholera toxin-B subunit. *J Biol Chem* 252: 7249–7256.

Leong, J., Vinal, A.C., and Dallas, W.S. (1986) Nucleotide sequence comparison between heat-labile enterotoxin B subunit cistrons for *Escherichia coli* of human and porcine origin. *Infect Immun* 48: 73–77.

Ludwig, D.S., Holmes, R.K., and Schoolnik, G.K. (1985) Chemical and immunochemical studies on the receptor binding domain of cholera toxin B subunit. *J Biol Chem* 260: 12528–12534.

Macintyre, S., and Henning, U. (1990) The role of the mature part of secretory proteins in translocation across the plasma membrane and in regulation of their synthesis in *Escherichia coli*. *Biochimie* **72**: 157–167.

Maneval, Jr, D.R., Colwell, R.R., Joseph, S.W., Gray, R., and Donta, S.T. (1980) A tissue culture method for the detection of bacterial enterotoxins. *J Tiss Cult Meth* **6**: 85–90.

Mattingly, Jr, J.R., Martinez-Carrion, M. (1990) Site-specific mutagenesis in the active site of aspartate aminotransferase: the effects of replacing tryptophan 140 with phenylalanine or glycine. *Ann NY Acad Sci* **585**: 526–528.

Mekalanos, J.J., Swartz, D.J., Pearson, G.D.N., Harford, N., Groyne, T., and DeWilde, M. (1983) Cholera toxin genes — nucleotide sequence, deletion analysis and vaccine development. *Nature* **306**: 551–557.

Miller, J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Miller, V.L., and Mekalanos, J.J. (1984) Synthesis of cholera toxin is positively regulated at the transcriptional level by *toxR*. *Proc Natl Acad Sci USA* **81**: 3471–3475.

Morelle, G. (1989) A plasmid extraction procedure on a miniprep scale. *Focus (BRL)* **11**: 7–8.

Mullin, B.R., Aloj, S.M., Fishman, P.H., Lee, G., Kohn, D., and Brady, B.O. (1976) Cholera toxin interactions with thyrotropin receptors on thyroid plasma membranes. *Proc Natl Acad Sci USA* **73**: 1679–1683.

Olsvik, O., Lund, A., Berdal, B., and Bergan, T. (1983) Differences in binding to the GM1 receptor by heat-labile enterotoxin of human and porcine *Escherichia coli* strains. *NIPH Annals* **6**: 5–14.

Olsnes, S., Stenmark, H., Moskaug, J.O., McGill, S., Madshus, I.H., and Sandvig, K. (1990) Protein toxins with intracellular targets. *Microb Pathogen* **8**: 163–168.

Pickett, C.L., Weinstein, D.L., and Holmes, R.K. (1987) Genetics of type IIa heat-labile enterotoxin of *Escherichia coli*: operon fusions, nucleotide sequence, and hybridization studies. *J Bacteriol* **169**: 5180–5187.

Pickett, C.L., Twiddy, E.M., Coker, C., and Holmes, R.K. (1989) Cloning, nucleotide sequence, and hybridization studies of the type IIb heat-labile enterotoxin gene of *Escherichia coli*. *J Bacteriol* **171**: 4945–4952.

Randall, L.L., and Hardy, S.J.S. (1986) Correlation of competence for export with lack of tertiary structure of the mature species: a study *in vivo* of the maltose-binding protein in *E. coli*. *Cell* **46**: 921–928.

Ribi, H.O., Ludwig, D.S., Mercer, K.L., Schoolnik, G.K., and Kornberg, R.D. (1988) Three dimensional structure of cholera toxin penetrating a lipid membrane. *Science* **239**: 1272–1276.

Russel, M., Kidd, S., and Kelley, M. (1986) An improved filamentous helper phage for generating single stranded plasmid DNA. *Gene* **45**: 333–338.

Sandkvist, M., Hirst, T.R., and Bagdasarian, M. (1990) Minimal deletion of amino acids from the carboxyl terminus of the B subunit of heat-labile enterotoxin causes defects in its assembly and release from the cytoplasmic membrane of *Escherichia coli*. *J Biol Chem* **265**: 15239–15244.

Schagger, H., and Jagow, G.V. (1987) Tricine SDS–PAGE for the separation of proteins in the range from 1–100 kDa. *Anal Biochem* **166**: 368–379.

Sheen, J.Y., and Seed, B. (1988) Electrolytic gradient gels for DNA sequencing. *BioTechniques* **6**: 943–944.

Spangler, B.D., and Westbrook, E.M. (1989) Crystallization of isoelectrically homogeneous cholera toxin. *Biochem* **28**: 1333–1340.

Surewicz, W.K., Leddy, J.J., and Mantsch, H.H. (1990) Structure, stability, and receptor interaction of cholera toxin as studied by fourier-transform infrared spectroscopy. *Biochem* **29**: 8106–8111.

Tabor, S., and Richardson, C.C. (1985) A bacteriophage T7 RNA polymerase/promoter system for the controlled exclusive expression of specific genes. *Proc Natl Acad Sci USA* **82**: 1074–1078.

Takao, T., Watanabe, H., and Shimonishi, Y. (1985) Facile identification of protein sequences by mass spectrometry B subunit of *Vibrio cholerae* classical biotype Inaba 569B toxin. *Eur J Biochem* **146**: 503–508.

Tsuji, T., Honda, T., Miwatani, T., Wakabayashi, S., and Matsubara, H. (1985) Analysis of receptor-binding site in *Escherichia coli* enterotoxin. *J Biol Chem* **260**: 8552–8558.

Tsuji, T., Iida, T., Honda, T., Miwatani, T., Nagahama, M., Sakurai, J., Wada, K., and Matsubara, H. (1987) A unique amino acid sequence of the B subunit of a heat-labile enterotoxin isolated from a human enterotoxigenic *Escherichia coli*. *Microb Pathogen* **2**: 381–390.

Tsuji, T., Fukuyama, K., Matsubara, H., Honda, T., and Miwatani, T. (1989) Crystallization and preliminary X-ray diffraction study of the heat-labile enterotoxin B subunit produced by enterotoxigenic *Escherichia coli*. *J Mol Biol* **208**: 207–208.

Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**: 103–119.